DRINKING WATER DISINFECTION BY-PRODUCTS: TOXICOLOGICAL IMPACTS AND BIOLOGICAL MECHANISMS INDUCED BY INDIVIDUAL COMPOUNDS OR AS COMPLEX MIXTURES

BY

CLARA H. JEONG

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Crop Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Professor Michael J. Plewa, Chair
Professor Benito J. Mariñas
Professor Jodi Flaws
Professor Susan R. Richardson, University of South Carolina

ABSTRACT

The introduction of drinking water disinfection greatly reduced the incidence of waterborne diseases. However, the reaction between disinfectants and natural or synthetic organic matter in the source water can lead to an unintended consequence which is the formation of drinking water disinfection by-products (DBPs). Many DBPs are mutagenic, cytotoxic, genotoxic, carcinogenic, or teratogenic. Studies on the molecular mechanisms of toxic effects induced by DBPs, and the overall toxicity of DBP complex mixtures are limited. The objectives of this dissertation were to i) analyze the occurrence and comparative toxicity of the emerging haloacetaldehyde (HAL) DBPs, ii) investigate the molecular mechanism of DBP-induced toxicity by the haloacetic acids (HAAs), iii) develop a single well microplate-based ATP-protein assay as a novel toxicity metric for DBPs, iv) investigate the occurrence and *in vitro* mammalian cell toxicity of DBPs in European drinking water samples collected from the site where epidemiological studies on reproductive outcomes were being conducted (HIWATE), and v) determine the impact of iodinated X-ray contrast media (ICM) in the source water and the type of disinfectant on the overall toxicity of DBP mixtures. From this study, iodoacetaldehyde was identified as a new DBP and ten HALs induced *in vitro* cytotoxicity and genotoxicity in Chinese hamster ovary (CHO) cells. HALs were the second most cytotoxic DBP class among six DBP chemical classes reported in the literature. Three mono-halogenated HAAs (monoHAA) including chloroacetic acid, bromoacetic acid, and iodoacetic acid induced ATP depletion in CHO cells, and cellular ATP levels was recovered when they were simultaneously treated with pyruvate. The magnitude of monoHAA-mediated ATP depletion highly correlated with the

monoHAA-induced inhibition kinetics of GAPDH and with diverse measurements of toxicity including cytotoxicity, genotoxicity, mutagenicity and teratogenicity published in the literature. A novel single well microplate-based ATP-protein assay was developed and with this assay, monoHAAs showed the greatest reduction in ATP levels while diHAAs showed a moderate reduction with higher concentration ranges. TriHAAs induced increases in ATP levels. The occurrence and *in vitro* mammalian cell toxicity of DBPs in the HIWATE study was investigated. The cytotoxic potency index values significantly correlated with the total number of identified DBPs and also with the concentration of 21 target DBPs. The genotoxic potency index values were not correlated with either of these metrics or with any DBP chemical class. In the ICM study, Iopamidol generated an enhanced level of CHO cell cytotoxicity and genotoxicity after disinfection, and the relative Iopamidol-mediated increase in toxicity was greater when chloramines was used as the disinfectant compared with chlorine. Four other ICMs (Iopromide, Iohexol, Diatrizoate and Iomeprol) expressed some cytotoxicity over the control, and expressed higher cytotoxicity when chlorinated. Only Iohexol enhanced genotoxicity compared to the chlorinated source water.

ACKNOWLEDGEMENT

"... He said to me, "My grace is sufficient for you, for my power is made perfect in weakness."
Therefore, I will boast all the more gladly about my weaknesses,
so that Christ's power may rest on me. - 2 Corinthians 12:9 - "

Heavenly Father,
I give you thanks with all my heart. You are my solid rock, my fortress and my shepherd.

My Ph.D. journey would not have been possible without the support and encouragement of so many people around me. First of all, I would like to express my deepest appreciation to my advisor, Professor Michael Plewa for his inspiration and guidance throughout my graduate study. I thank Dr. Elizabeth Wagner for all her help and guidance in the laboratory. I would also like to thank my doctoral committee members, Professor Benito Mariñas, Professor Jodi Flaws and Professor Susan Richardson, for their intellectual comments and encouragement. I thank my friends and colleagues in Dr. Plewa's laboratory group. Thank you, Yukako Komaki, Justin Pals, Jennifer Osiol and Azra Dad for your thoughtful comments, discussions and encouragements and for being a wonderful friend for me.

I am very thankful to my family for their unending love and care. Mom, Dad, and Michaela, I love you all so much.

I appreciate support by WaterCAMPWS, a Science and Technology Center for Advanced Materials for the Purification of Water with Systems, the NIEHS Pre-doctoral Training Fellowship in Endocrine, Developmental and Reproductive Toxicology, an international collaborative NSF research grant and finally, a U.S. EPA STAR grant.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1. DRINKING WATER DISINFECTION BY-PRODUCTS

The introduction of water disinfection greatly reduced the incidence of waterborne diseases such as cholera and typhoid and is considered a major public health achievement of the 20th century [1]. Chlorine is the most common disinfectant used worldwide, and alternatives include ozone, chloramines, chlorine dioxide, and UV radiation [2-4]. However, the reaction between disinfectants and natural organic as well as inorganic matter in the water can lead to an unintended consequence which is the formation of drinking water disinfection by-products (DBPs). The spectrum of DBP formation depends on many factors such as the source water characteristics, pH, temperature, type of disinfectant, and residence time [5-8].

Trihalomethanes (THMs) were the first DBP chemical class discovered in 1974 [9], and to date, more than 600 DBPs have been identified in finished drinking waters [6, 10]. Identified chemical classes of DBPs include THMs, haloacetic acids (HAAs), haloketones (HKs), haloacetaldehydes (HALs) and nitrogen-containing DBPs such as haloacetonitriles (HANs), haloacetamides (HAcAms) and hlaonitromethanes (HNMs). However, the chemical identification of the majority of DBPs, as the total organic halide in disinfected water, are unknown (Figure 1.1) [11]. While most of the DBP occurrence studies were conducted in U.S. and Canada, relatively little research has been published in Europe [12].

In 2006, the U.S. EPA issued the Stage 2 Disinfectants (D)/DBP Rule to control the maximum contaminant levels (MCLs) of certain DBPs. Currently, 11 DBPs are regulated in the United States (4 THMs; MCL = 80 μg/L, 5 HAAs; MCL = 60 μg/L, Bromate, BrO_3^-; MCL = 10 μg/L, Chlorite, ClO_2^-; MCL = 1.0 mg/L) [13]. Regulations of total THM levels have been established with various ranges worldwide (Austria, Belgium, Italy; MCL = 30 μg/L, Germany, Luxembourg, Sweden; MCL = 50 μg/L, Taiwan; MCL = 80 μg/L, China, Japan, Norway, Spain, and United Kingdom; MCL = 100 μg/L) [14].

The high dose *in vivo* bioassay results from National Institute of Cancer (NCI) indicated that THMs are carcinogenic [15] which generated public health concerns. Epidemiological studies demonstrated associations between DBPs in chlorinated water and an increased cancer risk such as bladder cancer, stomach cancer, and colon cancer [16-20]. Evidence on the association between DBPs and adverse pregnancy outcomes including spontaneous abortion, low birth weight (LBW), small-for-gestational-age (SGA), still birth, and preterm delivery were also found [21-24].

During the past decades, Plewa *et al.* established the first *in vitro* quantitative, systematic comparative studies on chronic cytotoxicity and genotoxicity of DBPs in mammalian cells (Figure 1.2 and Figure 1.3) [25, 26]. The cytotoxicity was measured by the reduction in cell density as a function of the DBP concentration over a period of 72 h (~3 cell cycles). The single cell gel electrophoresis (SCGE, or Comet) assay was used as the genotoxicity metric, which quantitatively measures genomic DNA damage in individual nuclei (Figure 1.3). These studies provided a direct comparison of the adverse biological impact of DBPs within DBP chemical

classes and identified those DBPs that were of highest concern for public health risks. These databases also provide guidelines to elucidate the structure-based mechanisms of DBPs toxicity. Based on the current results, iodinated and brominated DBPs in general, were shown to be more toxic than their chlorinated analogues [27-30]. Similarly, nitrogen-containing DBPs showed greater cytotoxicity and genotoxicity than carbon-containing DBPs [31]. These comparative databases continued to expand to include emerging DBPs such as haloacetonitriles (HANs) [26], haloacetamides (HAcAms) [27], hlaonitromethanes (HNMs) [29] and haloacetaldehydes (HALs) which are frequently detected in finished drinking water.

Our laboratory is investigating the mode of action of DBP toxicity. We discovered that the haloacetic acids (HAAs) inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which is a primary controlling enzyme in glycolysis. When HAAs inhibit GAPDH activity, glycolysis is blocked resulting in decrease in pyruvate which is required by the tricarboxylic acid (TCA) cycle in the mitochondria. The disruption of the electron transport chain in the TCA cycle induces the generation of reactive oxygen species (ROS). Recently, it was found that the mono-halogenated HAAs (monoHAAs) inhibit GAPDH activity in a concentration-dependent manner [32] . The inhibition rate of GAPDH activity and the toxic potency of the monoHAAs were highly correlated. The inhibition kinetics showed a strong correlation with other toxicological endpoints which were previously published [32]. Also, the HAA induced expression of many genes involved in ROS response [33]. The quantitative analyses to investigate the roles of pyruvate on ATP generation and ROS production is a goal of our laboratory.

While investigating the toxicity of a single chemical is important, the toxicological effects of a complex DBP mixture should be considered carefully because the complex mixture represents the composition of finished water that people consume in daily life. Most correlations in published epidemiology studies are based on the level of total THM, and the level of other DBPs are often not considered [23, 24, 34, 35]. These studies may overlook the effect of emerging DBPs which may possess higher toxic potencies. In 2009, the European Union (EU) started a major research initiative named HIWATE (Health Impacts of long-term exposure to disinfection by-products in drinking WATEr) to investigate the occurrence and potential human health risks associated with long-term exposure to low levels of DBPs in drinking water [36]. During the February 2010 HIWATE Scientific Advisory Committee meeting in London, it was proposed to augment the study to include comprehensive analyses of the analytical chemistry and the analytical *in vitro* toxicology of the HIWATE drinking water samples collected from different distribution systems, where an epidemiologic study of reproductive outcomes was being conducted. This proposal became the foundation of a component of this dissertation.

Among various factors influencing the spectrum of DBPs in finished water, the source water characteristics may play an important role, especially in a local site where large amount of industrial or medical contaminants were being discharged. For example, Iodinated X-ray contrast media (ICM) are widely used at medical centers for soft tissues medical imaging such as organs, veins, and blood vessels. The worldwide consumption of ICM is around 3.5×10^6 kg/year, and a single application can be up to 200g/d [37]. Incomplete removal of ICMs in wastewater treatment plants could lead to an elevation of ICM concentrations in streams and

rivers [38-41]. ICM are primary contributors to the total organic halogen burden in clinical wastewater and play a major role as a source of absorbable organic iodine in wastewater [42]. ICM may be good sources of iodine to form iodinated DBPs (iodo-DBPs), and the activated benzene rings and other functional groups that can react with oxidizing disinfectants may lead ICM to be potential DBP precursors. An international collaboration study (U.S. and Germany) is ongoing on the impacts of ICM on DBP formation and toxicity. The general hypothesis is that oxidizing disinfectants such as chlorine and chloramine react with ICM to form highly toxic iodo-DBPs and higher molecular weight by-products of unknown toxicity. This study proposed to carry out controlled laboratory reactions of 5 ICMs (Iopamidol, Diatrizoate, Iopromide, Iomeprol, and Iohexol) with chlorine and chloramine under different condition to simulate drinking water treatment. In this dissertation, the mammalian cell cytotoxicity and genotoxicity of the reaction product mixtures from two experimental designs were measured to determine which ICM and reaction conditions give rise to toxicity, as a part of the overall project.

1.2. RESEARCH OBJECTIVES

The overall research objective is to analyze the comparative toxicity of emerging haloacetaldehyde DBPs and to investigate the molecular mechanism of DBP induced toxicity with HAAs. Development of a single well microplate-based ATP-protein measurement assay with HAAs as a novel toxicity metric for DBPs is included as a part of the research. In addition to individual DBP studies, two DBP mixture studies are included in the dissertation. First study investigated the occurrence and *in vitro* mammalian cell toxicity of DBPs in European drinking

water samples collected from the site where the epidemiology studies on reproductive outcomes are being conducted in relation with the HIWATE project. The other DBP mixture study focuses on the impact of ICM in the source water and the type of disinfectant on the overall toxicity of DBP mixtures. Specific research objectives are summarized below:

1. The occurrence and comparative toxicity of the haloacetaldehyde disinfection by-products in drinking water

- Develop new analytical chemical methods and identify the occurrence of a new haloacetaldehyde DBP in drinking water (In collaboration with Dr. Richardson and Dr. Postigo).
- Analyze the *in vitro* cytotoxicity and genotoxicity of HALs and related compounds in mammalian cells.
- Determine the cytotoxicity and genotoxicity index values of HALs and develop a quantitative, comparative toxicity database.
- Conduct a mechanism-based structure-activity relationship analysis for the observed HAL mediated cytotoxicity and genotoxicity.

2. Investigate the biological mechanism induced by haloacetic acid disinfection by-products and the development of a single well microplate-based ATP-protein measurement assay

- Measure the impact of the monoHAAs on ATP levels with and without pyruvate supplementation.

- Develop a new single well microplate-based ATP-protein measurement assay with HAAs as a novel molecular toxicity metric for DBPs.

3. The occurrence and toxicity of disinfection by-products in European drinking waters in relation with the HIWATE epidemiology study

- Obtain disinfected drinking water from HIWATE cities, extract and concentrate the organic fractions and chemically analyze for DBPs (in collaboration with Dr. S. Richardson).
- Determine the relative chronic cytotoxicity and acute genotoxicity in mammalian cells for each HIWATE sample.
- Analyze for correlations between the toxicity data and the occurrence and concentrations of DBPs.

4. The impact of X-ray contrast agents on formation and toxicity of disinfection by-products in drinking water

- Generate XAD resin concentrates of source water treated with ICMs and disinfectants (in collaboration with Dr. S. Duirk).
- Measure the mammalian cell cytotoxicity and genotoxicity of the reaction product mixtures.
- Determine which ICM and reaction conditions give rise to toxic by-products.

References

1. Cutler, D.; Miller, G., The role of public health improvements in health advances: the twentieth-century United States. *Demography* **2005,** *42*, (1), 1-22.
2. Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Res.* **2007,** *41*, (8), 1667-1678.
3. Reckhow, D. A.; Linden, K. G.; Kim, J.; Shemer, H.; Makdissy, G., Effect of UV treatment on DBP formation. *J. Am.Water Works Assoc.* **2010,** *102*, (6), 100-113.
4. Zhang, X.; Echigo, S.; Minear, R. A.; Plewa, M. J., Characterization and comparison of disinfection by-products of four major disinfectants. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 299-314.
5. Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. *Philos. Transact. A Math. Phys. Eng. Sci.* **2009,** *367*, (1904), 4077-4095.
6. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636*, (1-3), 178-242.
7. Ueno, H.; Moto, T.; Sayato, Y.; Nakamuro, K., Disinfection by-products in the chlorination of organic nitrogen compounds: by-products from kynurenine. *Chemosphere* **1996,** *33*, (8), 1425-1433.
8. Ye, B.; Wang, W.; Yang, L.; Wei, J.; E, X., Formation and modeling of disinfection by-products in drinking water of six cities in China. *J. Environ. Monit.* **2011,** *13*, (5), 1271-1275.
9. Rook, J. J., Formation of haloforms during chlorination of natural waters. *J. Soc. Water Treat. Exam.* **1974,** *23*, 234-243.
10. Richardson, S. D., Disinfection by-products: formation and occurrence in drinking water. In *Encyclopedia of Environmental Health*, Nriagu, J. O., Ed. Elsevier: Burlington, 2011; Vol. 1, pp 110-136.
11. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., The occurrence of a new generation of disinfection by-products. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-7185.
12. Goslan, E. H.; Krasner, S. W.; Bower, M.; Rocks, S. A.; Holmes, P.; Levy, L. S.; Parsons, S. A., A comparison of disinfection by-products found in chlorinated and chloraminated drinking waters in Scotland. *Water Res.* **2009,** *43*, (18), 4698-4706.
13. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 disinfectants and disinfection byproducts rule. *Fed. Reg.* **2006,** *71*, (2), 387-493.
14. Karanfil, T.; Krasner, S. W.; Westerhoff, P.; Xie, Y., Recent advances in disinfection by-product formation, occurrence, control, health effects, and regulations. In *Disinfection by-products in drinking water*, Karanfil, T.; Krasner, S. W.; Westerhoff, P.; Xie, Y., Eds. American Chemical Society, Washington, D.C., 2008.

15. NCI *Carcinogensis bioassay of chloroform*; National Cancer Institute: Bethesda, MD, 1976.
16. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286*, (1-3), 1-19.
17. Costet, N.; Villanueva, C. M.; Jaakkola, J. J.; Kogevinas, M.; Cantor, K. P.; King, W. D.; Lynch, C. F.; Nieuwenhuijsen, M. J.; Cordier, S., Water disinfection by-products and bladder cancer: is there a European specificity? A pooled and meta-analysis of European case-control studies. *Occup. Environ. Med.* **2011,** *68*, (5), 379-385.
18. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39*, (3), 733-745.
19. Villanueva, C. M.; Cantor, K. P.; Cordier, S.; Jaakkola, J. J.; King, W. D.; Lynch, C. F.; Porru, S.; Kogevinas, M., Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* **2004,** *15*, (3), 357-367.
20. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.
21. Grellier, J.; Bennett, J.; Patelarou, E.; Smith, R. B.; Toledano, M. B.; Rushton, L.; Briggs, D. J.; Nieuwenhuijsen, M. J., Exposure to disinfection by-products, fetal growth, and prematurity: a systematic review and meta-analysis. *Epidemiology* **2010,** *21*, (3), 300-313.
22. Hinckley, A. F.; Bachand, A. M.; Reif, J. S., Late pregnancy exposures to disinfection by-products and growth-related birth outcomes. *Environ. Health Perspect.* **2005,** *113*, (12), 1808-1813.
23. Wright, J. M.; Schwartz, J.; Dockery, D. W., Effect of trihalomethane exposure on fetal development. *Occup. Environ. Med.* **2003,** *60*, (3), 173-180.
24. Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ. Res.* **2007,** *104*, (3), 390-395.
25. Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2002,** *40*, (2), 134-142.
26. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
27. Plewa, M. J.; Muellner, M. G.; Richardson, S. D.; Fasano, F.; Buettner, K. M.; Woo, Y. T.; McKague, A. B.; Wagner, E. D., Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: an emerging class of nitrogenous drinking water disinfection byproducts. *Environ. Sci. Technol.* **2008,** *42*, (3), 955-961.

28. Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2010,** *51*, (8-9), 871-878.
29. Plewa, M. J.; Wagner, E. D.; Jazwierska, P.; Richardson, S. D.; Chen, P. H.; McKague, A. B., Halonitromethane drinking water disinfection byproducts: chemical characterization and mammalian cell cytotoxicity and genotoxicity. *Environ. Sci. Technol.* **2004,** *38*, (1), 62-68.
30. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D., Jr.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38*, (18), 4713-4722.
31. Plewa, M. J.; Wagner, E. D.; Muellner, M. G.; Hsu, K. M.; Richardson, S. D., Comparative mammalian cell toxicity of N-DBPs and C-DBPs. In *Occurrence, formation, health effects and control of disinfection by-products in drinking water*, Karanfil, T.; Krasner, S. W.; Westerhoff, P.; Xie, Y., Eds. American Chemical Society: Washington, D.C., 2008; Vol. 995, pp 36-50.
32. Pals, J. A.; Ang, J. K.; Wagner, E. D.; Plewa, M. J., Biological mechanism for the toxicity of haloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2011,** *45*, (13), 5791-5797.
33. Pals, J.; Attene-Ramos, M. S.; Xia, M.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47*, (21), 12514-12523.
34. Grazuleviciene, R.; Nieuwenhuijsen, M. J.; Vencloviene, J.; Kostopoulou-Karadanelli, M.; Krasner, S. W.; Danileviciute, A.; Balcius, G.; Kapustinskiene, V., Individual exposures to drinking water trihalomethanes, low birth weight and small for gestational age risk: a prospective Kaunas cohort study. *Environ. Health* **2011,** *10*, 32.
35. Villanueva, C. M.; Gracia-Lavedan, E.; Ibarluzea, J.; Santa Marina, L.; Ballester, F.; Llop, S.; Tardon, A.; Fernandez, M. F.; Freire, C.; Goni, F.; Basagana, X.; Kogevinas, M.; Grimalt, J. O.; Sunyer, J.; Project, I., Exposure to trihalomethanes through different water uses and birth weight, small for gestational age, and preterm delivery in Spain. *Environ. Health Perspect.* **2011,** *119*, (12), 1824-1830.
36. Nieuwenhuijsen, M. J.; Smith, R.; Golfinopoulos, S.; Best, N.; Bennett, J.; Aggazzotti, G.; Righi, E.; Fantuzzi, G.; Bucchini, L.; Cordier, S.; Villanueva, C. M.; Moreno, V.; La Vecchia, C.; Bosetti, C.; Vartiainen, T.; Rautiu, R.; Toledano, M.; Iszatt, N.; Grazuleviciene, R.; Kogevinas, M., Health impacts of long-term exposure to disinfection by-products in drinking water in Europe: HIWATE. *J. Water Health* **2009,** *7*, (2), 185-207.
37. Perez, S.; Eichhorn, P.; Celiz, M. D.; Aga, D. S., Structural characterization of metabolites of the X-ray contrast agent iopromide in activated sludge using ion trap mass spectrometry. *Anal. Chem.* **2006,** *78*, (6), 1866-1874.
38. Carballa, M.; Omil, F.; Lema, J. M.; Llompart, M.; Garcia-Jares, C.; Rodriguez, I.; Gomez, M.; Ternes, T., Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* **2004,** *38*, (12), 2918-2926.

39. Kormos, J. L.; Schulz, M.; Kohler, H. P.; Ternes, T. A., Biotransformation of selected iodinated X-ray contrast media and characterization of microbial transformation pathways. *Environ. Sci. Technol.* **2010,** *44*, (13), 4998-5007.
40. Putschew, A.; Schittko, S.; Jekel, M., Quantification of triiodinated benzene derivatives and X-ray contrast media in water samples by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* **2001,** *930*, (1-2), 127-134.
41. Schittko, S.; Putschew, A.; Jekel, M., Bank filtration: a suitable process for the removal of iodinated X-ray contrast media? *Water Sci. Technol.* **2004,** *50*, (5), 261-268.
42. Kummerer, K.; Erbe, T.; Gartiser, S.; Brinker, L., AOX-emissions from hospitals into municipal waste water. *Chemosphere* **1998,** *36*, (11), 2437-2445.

FIGURES

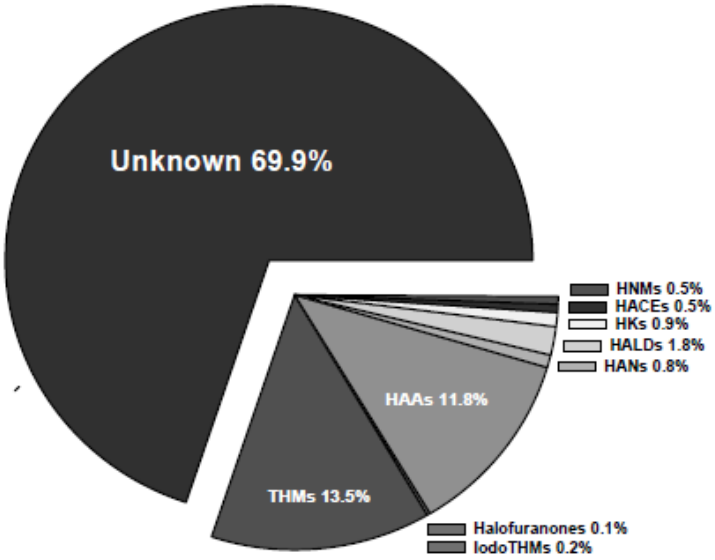


Figure 1.1. Summary of the distribution of median values of the DBP chemical classes in water analyzed in the U.S. EPA Nationwide Occurrence Study as a component of total organic halides [11].

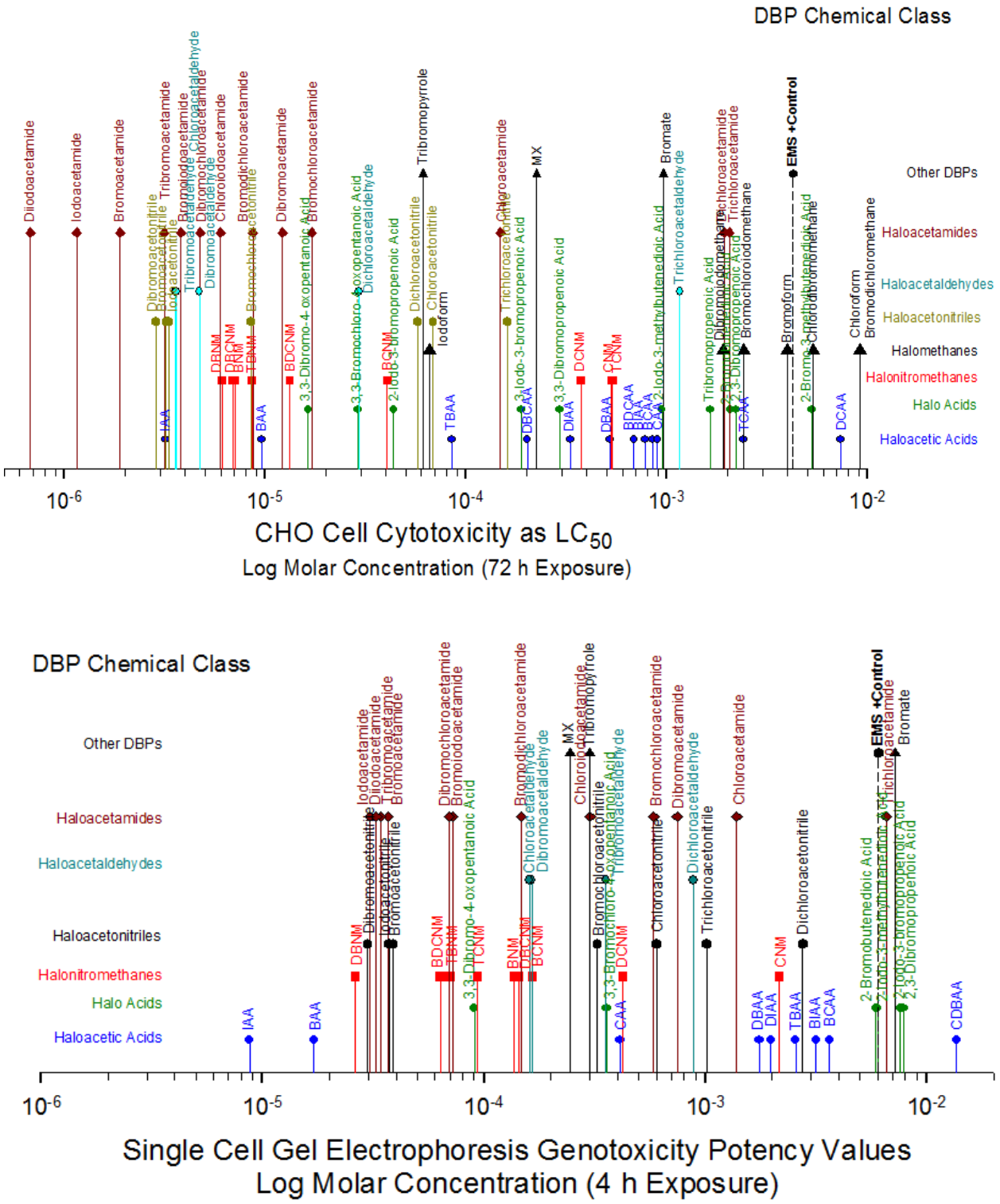


Figure 1.2. Comparative CHO cell DBP chronic cytotoxicity database (above) and comparative CHO cell DBP acute genotoxicity database (below) conducted by Plewa and Wagner [26].

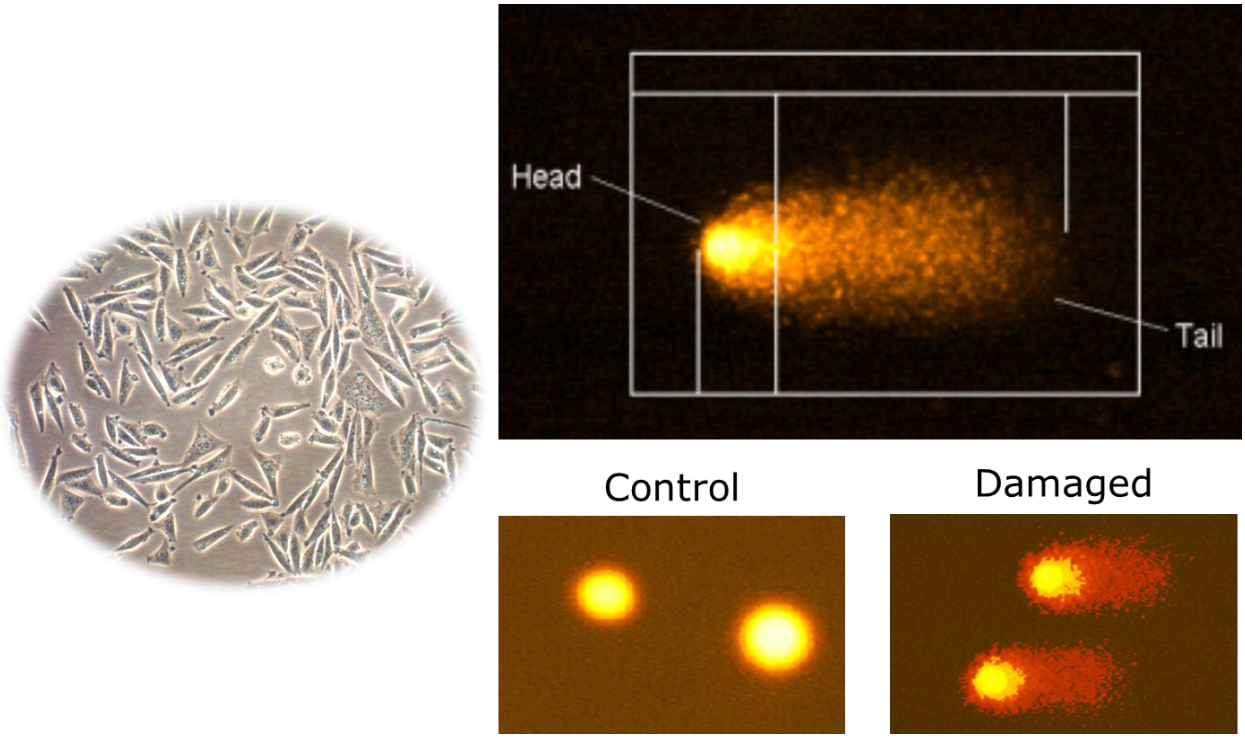


Figure 1.3. Chinese hamster ovary (CHO) cell line AS 52, clone 11-4-8 (left). Images of single cell gel electrophoresis (SCGE) assay illustrating genomic DNA damage in a nucleus (right).

CHAPTER 2

LITERATURE REVIEW

2.1. HALOACETALDEHYDE DISINFECTION BY-PRODUCTS

2.1.1. Occurrence

Aldehyde DBPs are mainly formed through the use of ozone disinfectant where the typical levels of the sum of halogenated and non-halogenated aldehyde DBPs are 5 to 20 μg/L [1]. In 1996, the U.S. EPA Information Collection Rule (ICR) was announced, which the purpose was to collect information on the occurrence and control of microbial pathogens and DBPs in drinking water [2]. A large survey was conducted in 296 U.S. water utilities, which operated about 500 drinking water plants, including surface water systems and groundwater systems. The survey measured 4 THMs, 6 haloacetic acids (HAAs), and 6 other halogenated DBPs in the treatment plants and in the distribution systems. In this report, haloacetaldehdyes (HALs) were found to be at higher concentrations in water treatment systems using ozone than chlorine dioxide (up to 30.6 μg/L) [2].

Within the HAL DBP class, chloral hydrate (trichloroacetaldehyde, TCAL) is a major HAL identified. TCAL is primarily a chlorine or chloramine generated DBP, and the use of ozone prior to chlorination or chloramination is known to increase its formation [3]. According to the ICR, TCAL was detected to be at higher levels in the distribution system (median 2.8 μg/L; 90th percentile 11.0 μg/L) than in the finished water (median 1.7 μg/L; 90th percentile 7.4 μg/L) [2].

Chloroacetaldehyde (CAL) and dichloroacetaldehyde (DCAL) are formed in chlorinated drinking water. However, information on their occurrence is limited because they can easily be transformed into TCAL in water [1]. Previously, the occurrence of brominated HALs was not well reported in drinking water analysis because of the lack of commercial standards. Standards of bromochloroacetaldehyde (BCAL), dibromoacetaldehyde (DBAL), bromodichloroacetaldehyde (BDCAL), dibromochloroacetaldehyde (DBCAL) and tribromoacetaldehyde (TBAL) have been made commercially available recently. Brominated HALs are generated when bromide containing waters are chlorinated [4].

In 2006, a nationwide occurrence study was published by Krasner *et al.* [3]. They evaluated the effect of source water and treatment conditions on DBP formation and identified emerging DBPs. CAL, DCAL, BCAL, and TCAL were included in this study as priority DBPs [3]. In this study, HALs were the third largest DBP class by weight of all the DBPs studied and DCAL was the most abundant, with a maximum concentration of 16 µg/L in a simulated distribution system sample [3].

In early occurrence studies in Canadian drinking water, TCAL was detected in most chlorinated water samples [5, 6]. In addition, recent studies have found most target HALs in drinking water, while the speciation depends on water parameters such as bromide concentration and treatment processes [4, 7]. Koudjonou *et al.* assessed various Canadian drinking water supplies and collected samples at the treatment plant and at the distribution system [4, 7]. In their study, TCAL/THMs ratios (w/w) significantly varied with the type of disinfectant. TCAL/THMs ratios were the lowest (4-22%) in chloraminated waters, and were the

highest (12-52%) when ozone was applied as the pre- or primary disinfectant, prior to post chlorination. These results indicated that the ozone followed by post-chlorination enhanced the formation of TCAL in waters. Ozone produced increased levels of oxygenated DBPs including acetaldehyde, which is a potential precursor of other chlorinated HAL DBPs [8, 9]. It was observed that the contribution of TCAL to the total chlorinated DBPs increased in cold water [7]. In waters containing bromide, brominated HALs were also detected after chlorination [7]. Currently, there is no Canadian guideline for TCAL in drinking water. The guideline for TCAL established by the WHO is 10 µg/L and the drinking water guideline in Australia and New Zealand is 20 µg/L.

In terms of seasonal and spatial variations, LeBel *et al.* conducted a one-year survey and found that the levels of TCAL was higher during the summer than in winter at the treatment plant and the early part of the distribution system [5]. In summer, the maximum TCAL levels were found within the distribution system and the minimum TCAL levels were found at the end-system locations, which suggest that TCAL is transformed to THM in warm water through long residence time [5].

2.1.2. Analytical Methods

Currently, two methods, EPA Method 556.1 and Standard Method 6252 are commonly used for HAL analysis [10, 11]. Both methods use O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBHA) to derivatize aldehydes to their pentafluorobenzyl oximes in aqueous phase. Their oxime derivatives are then extracted with hexane and analyzed by gas chromatography (GC)/electron capture detection (ECD) method.

EPA Method 556.1, a derivative of EPA Method 556, uses a fast gas chromatographic technique for oxime detection. Due to its poor extraction efficiency with pentane, for HAL analysis, MTBE is used as the extraction solvent, and stock solutions of the brominated standards (BCAL, BDCAL, DBAL, CDBAL, TBAL) need to be prepared in MTBE and kept refrigerated ($4^{o}C$) [4]. Since chloramines are known to cause degradation of TCAL during sample storage, ammonium chloride cannot be used for the preservation of TCAL [1]. The liquid-liquid extraction (LLE) with GC/ECD method for chlorinated DBPs was validated for the determination of other di- and tri-HALs in water, as new standards were made available [4, 11].

2.1.3. Toxicity of Haloacetaldehydes

2.1.3.1. Chloroacetaldehyde

Chloroacetaldehyde (CAL) is a major reactive metabolite of various industrial chemicals such as vinyl chloride, ethylene dichloride and ethylene chlorohydrin. Vinyl chloride is a well known carcinogen, which is metabolically activated by cytochrome P450 to chloroethylene oxide followed by a rearrangement to CAL [12]. Therefore, many scientists focused on the toxicity of CAL as a metabolite of these industrial materials. In 1965, Johnson measured the influence of several aliphatic compounds on rat liver glutathione (GSH) levels and found that an oral administration of 0.53 mmol/kg of CAL in female rats induced 55% reduction in the GSH level [13]. The metabolism of CAL has been studied both *in vitro* and *in vivo* by Guengerich *et al.* and it was found that horse liver alcohol dehydrogenase catalyses the NADH-dependent reduction of CAL to form 2-chloroethanol, while yeast aldehyde dehydrogenase catalyses the NAD+-dependent oxidation CAL to form 2-chloroacetic acid [12]. Ifosfamide, an anticancer drug,

undergoes side chain oxidation in humans to form CAL and recent studies focused on the toxicity of CAL as side effects of ifosfamide treatment [14]. Lind *et al.* found that CAL caused depletion of GSH in lymphocytes from patients receiving ifosfamide [15]. CAL was used as a fluorescent label during the 1970s in biochemical research [16, 17]. Kochetkov *et al.* investigated the interaction of CAL with nucleic acid components and suggested their observation as a basis for a method of nucleic acid modification [18]. In that study, CAL reacted readily with 9-N-methyladenine and N-methylcytosine in weakly acidic aqueous solutions.

The molecular mechanism involved in CAL-induced cytotoxicity was investigated by Sood and O'Brien [19, 20]. CAL induced a loss in isolated rat hepatocyte viability in a time- and concentration- dependent manner, followed by a steady decrease in protein thiol levels, mitochondrial respiration and transmembrane potential which resulted in ATP depletion and lipid peroxidation [19]. They suggested that cytotoxic CAL concentrations caused oxidative stress and that ATP levels can be restored if cellular redox homeostasis is normalized [20]. The effect of chloroacetaldehyde on intracellular Ca^{2+} homeostasis in human renal proximal tubule cells (RPTEC) in primary culture was investigated and CAL induced nephrotoxicity by inhibiting the Na^{+}/Ca^{2+} exchanger which is dependent on protein kinase A (PKA) [21]. CAL induced necrotic cell death rather than apoptotic cell death.

CAL was mutagenic in various models. CAL reacted with single-stranded DNA to produce cyclic etheno adducts (ε-adducts) including 3,N4-ethenocytosine (εC), 1,N6-ethenoadenosine (εA), N2,3-ethenoguanine, and 1,N2-ethenoguanine *in vitro* [22-24]. Biernat *et al.* observed the reaction of CAL with some tRNA constituents and found the $–CH_2CH/OH/-$ bridge between the

exo and endo nitrogen atoms of the parent compounds [25]. These ε-adducts caused mutations both *in vitro* and *in vivo* [26-33]. The ε-adducts decreased DNA synthesis [34]. Choi *et al.* observed that CAL preferentially induces C/G to T/A transition and C/G to A/T transversion mutations in mammalian cells and concluded that methylated CpG sites were not preferential targets for CAL-induced mutagenesis [35]. In a *Salmonella typhimurium* strain TA1530, CAL was a strong alkylating mutagenic agent [36]. In *S. typhimurium* strain TA100, CAL was hundreds of times more mutagenic than other metabolites of vinyl chloride or dichloroethane [37]. Guengerich *et al.* found that CAL irreversibly bound to microsomal protein under *in vitro* conditions when purified dehydrogenases were utilized [12, 38]. In Chinese hamster V79 cells, CAL caused a dose-dependent induction of 8-azaguanine and ouabain-resistant mutants *in vitro* [39]. CAL also induced mitotic chromosome malsegregation in *Aspergillus nidulans* [40].

Maciejewska *et al.* focused on the role of AlkAl, AlkB and Mug proteins, which are engaged in repair of CAL-induced ε-adducts [41]. In that study, pIF102 and pIF104 plasmids were CAL-damaged *in vitro* and replicated in *E. coli* of various genetic backgrounds with modified levels of AlkA and AlkB proteins. They showed that all *alkA, alkB* and *mug* genes were engaged in alleviation of CAL-induced mutagenesis, suggesting that the active site of AlkB protein interacts with the CAL induced ε-adducts to affect the repair of εC *in vivo*. In addition, CAL induced interstrand cross-links *in vitro* in salmon sperm DNA where the cross-links formation depended on the reaction time and CAA concentration [42].

Recently, the carcinogenicity of CAL was investigated. Daniel *et al.* found that the liver is the primary target organ in B6C3F1 mice when they were administered 0.1 g/L of CAL through

their drinking water [43]. In that study, hepatocellular necrosis, hepatocellular hyperplasia and chronic active inflammation were found in CAL treated mice along with the increased liver weight and liver tumor induction.

2.1.3.2. Trichloroacetaldehyde (Chloral Hydrate)

The toxicity of trichloroacetaldehyde (TCAL) is the most widely studied compound among HALs. The oral 50% lethal dose (LD_{50}) of TCAL in mice was reported to be 1442 mg/kg body weight (bw) in males and 1265 mg/kg bw in females, and the LD_{50} in rats was 480 mg/kg bw [44, 45]. TCAL was mutagenic in *S. typhimurium* [46-49] and induced chromosomal aberrations in mammalian cells [46, 50]. TCAL induced aneuploidy [51, 52] and micronuclei [53-56] in mammalian cells. Using a micronucleus assay with cultured human lymphocytes, Migliore *et al.* found that TCAL induced aneuploidogenic activity [57]. TCAL induced mitotic aberrations [56, 58-60], and DNA strand-breaks [14, 61, 62] in mammalian cells. Administration of TCAL for 90 days to male rats at a concentration of 2.4 mg/mL increased focal hepatocellular necrosis [43].

Several studies focused on the possible adverse reproductive and prenatal effects induced by TCAL. TCAL crosses the human placenta at term [63] and low levels of chloral hydrate were found in breast milk [63]. Lambert *et al.* suggested that prolonged administration of sedative dose of TCAL to newborns increases the likelihood of hyperbilirubinaemia [64]. TCAL induced spindle aberrations and metaphase I arrest in mouse oocytes [65]. TCAL or its metabolite in the testis induced spermatid micronuclei in mice where spermatogonial sperm-cell phase was significantly affected [53]. Male rats administered with 2 g/L TCAL in drinking

water for 52 weeks showed significantly decreased percentages of motile and progressively motile sperm [66]. A shift from a straight-line velocity distribution of sperm to a lower modal velocity range was observed as well.

2.1.3.3. Other Haloacetaldehydes

Crebelli *et al.* found that dichloroacetaldehyde (DCAL) induced mitotic aneuploidy in lower eukaryotes [40]. They examined several halogenated aliphatic hydrocarbons to test their ability to induce somatic segregation in *Aspergillus nidulans* and found that DCAL significantly increased the frequency of haploid sectors and diploid non-disjunctional sectors. Because commercial standards for most brominated HALs were not available until recently, limited studies were performed on brominated HALs. Bromoacetaldehyde (BAL) irreversibly bound to DNA and protein in rat liver microsomes [38]. Tribromoacetaldehyde (TBAL) induced single- and double-strand DNA breaks [14].

Most of these toxicological studies were conducted for one or two compounds and considered the HAL as a metabolite of a parent chemical of interest. Therefore, to gain better understanding on HAL toxicity and their relative health risks compared with other DBPs, a systematic, quantitative, comparative study on the toxicity of individual HALs was needed.

2.2. MOLECULAR MECHANIMS OF HALOACETIC ACID DISINFECTION BY-PRODUCTS INDUCED TOXICITY

2.2.1. Haloacetic Acid Disinfection By-products

Haloacetic acids (HAAs) DBPs are found in most disinfected drinking waters. Currently, five HAAs, chloroacetic acid (CAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromoacetic acid (BAA), and dibromoacetic acid (DBAA) are regulated by the U.S. EPA at a maximum contaminant level of 60 μg/L for the sum of them (Figure 2.1) [67]. Other unregulated HAAs include bromochloroacetic acid (BCAA), iodoacetic acid (IAA), tribromoacetic acid (TBAA), bromodichloroacetic acid (BDCAA), and chlorodibromoacetic acid (CDBAA). Based on the U.S. EPA's information collection rule record, the mean and 90^{th} percentile concentration for the regulated five HAAs from all water sites measured were 23 μg/L and 47.5 μg/L [2]. DCAA and TCAA were the dominant HAAs found in drinking water. Levels of brominated species (BCAA and DBAA) increased when high bromide levels (>50 μg/L) were present in the source waters [3, 68].

2.2.2. Toxicological Studies on Haloacetic Acids

HAAs are mutagenic in *S. typhimurium* and genotoxic in Chinese hamster ovary (CHO) cells and non-transformed human cells [69-73]. IAA induced chromosome aberration in CHO cells [74]. DCAA induced a weak chromosome breaks in mice and induced mutation and chromosome aberrations in mouse lymphoma cells [75, 76]. Monohalogenated HAAs (MonoHAAs) were teratogenic in mouse embryos [77, 78]. Hunter *et al.* found that monoHAAs induce dysmorphogenesis and affected neural tube development, eye development, and heart

development under *ex-vivo* conditions [77]. IAA induced malignant transformation in NIH/3T3 mouse embryonic fibroblast cells that progressed to aggressive fibrosarcomas when implanted in Balb/c nude mice [79]. Gestational exposure of mixtures of regulated five HAAs resulted in pregnancy loss and eye malformation in rats [80]. Dichloroacetic acid (DCAA), bromochloroacetic acid (BCAA), and dibromoacetic acid (DBAA) altered intestinal microflora and metabolism in rats which could further affect the bioactivation of promutagens or procarcinogens [81].

Since the HAAs are the most regulated chemical class of DBPs, many studies focused on the molecular basis of HAA toxicity as well. Toxicogenomic analyses using non-transformed human cells found that monoHAAs modulated the gene expressions involved in stress response to DNA damage, DNA repair, especially, the repair of double-strand breaks, and in cell cycle regulation [82, 83]. IAA induced toxicity in hippocampal neuronal cells by inhibiting the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which led to the generation of reactive oxygen species (ROS) and hypoglycemia [84]. Similar effects were found in hippocampal astrocytes [85].

Glycolysis is the major metabolic pathway that converts glucose into pyruvate, which consists of a series of enzyme-catalyzed steps. The free energy released during this process is used to form ATP and NADH. GAPDH is an enzyme involved in the sixth step of glycolysis, which catalyses the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. When the activity of GAPDH is inhibited, glycolysis is blocked resulting in a reduction of pyruvate which is required by the tricarboxylic acid (TCA) cycle in the mitochondria. A disruption of the

electron transport chain in the TCA cycle induces the generation of ROS. Pals *et al.* found that the monoHAAs inhibit GAPDH activity in a concentration-dependent manner [86]. The inhibition of GAPDH activity and the toxic potency of the monoHAAs were highly correlated and the inhibition kinetics showed a strong correlation with other toxicological endpoints which were previously published [86]. However, quantitative analyses to investigate the detailed mechanisms connecting pyruvate level, ATP generation and ROS production were needed.

2.3. EPIDEMIOLOGICAL STUDIES ON ADVERSE EFFECTS OF DISINFECTION BY-PRODUCT EXPOSURE

2.3.1. Cancer Risks

Overall, evidence for the induction of urinary bladder cancer has been the most consistent, indicating the association with chlorinated DBPs [87] (Table 2.1). Most of the studies used the total THM (TTHM) levels as an indicator of DBP exposure and showed an association of DBP exposure with bladder cancer, with few exceptions [88-90]. It is also notable that recent study results which involved better estimation of DBP exposure showed higher, and statistically significant odd ratios (ORs) compared to old studies. These continuing results are strongly supporting the public concerns on DBPs as a possible cancer risk factor.

On the other hand, the epidemiologic evidence on chlorination DBPs to an association with increased colon and rectal cancer risk is still inconclusive. King *et al.* conducted a population-based case-control study in Canada to access the relationship between chlorination by-products in public water supplies and colon and rectum cancers [91]. In that study, males

exposed to chlorinated surface water for 35-40 years had an increased risk of colon cancer compared to those exposed for < 10 years (OR=1.53, 95% confidence interval (CI) = 1.13-2.09). No relationship was observed for females and no relationship was observed between rectal cancer risk and exposure to chlorination by-products for both males and females. In a prospective cohort study conducted with postmenopausal women, it was found that women who were consistently in the high exposure categories had the highest risk of colon cancer (Relative ratio (RR) = 1.86, 95% Cl = 1.29-2.69) [89]. Rahman *et al.* conducted a meta-analysis with a total of 13 studies (3 cohort and 10 case-control) and the pooled RR estimates of colon cancer were 1.11 (95% CI = 0.73-1.70) for cohort studies, 1.33 (95% CI = 1.12-1.57) for case-control studies and 1.27 (95% CI = 1.08-1.50) for all studies. For rectal cancer, the corresponding RR estimates were 0.88 (95% CI = 0.57-1.35), 1.40 (95% CI = 1.15-1.70) and 1.30 (95% CI = 1.06-1.59) [92].

2.3.2. Adverse Pregnancy Outcomes

The summary of epidemiological studies on adverse pregnancy outcomes associated with DBP exposure is presented in Table 2.2. Compared to the studies on cancer risk, studies on adverse pregnancy outcomes have broader endpoints and tend to have lower ORs. Hwang *et al.* conducted a cross-sectional study to evaluate the risks of birth defects according to four chlorination by-product exposure categories on the basis of chlorination (yes/no) and level of water color, which represents the amount of natural organic matter. The risks of any birth defect (OR = 1.13, 95% CI = 1.01-1.25), cardiac defect (OR = 1.37, 95% CI = 1.00-1.89), respiratory system defect (OR = 1.89, 95% CI = 1.00-3.58), and urinary tract defect (OR = 1.46,

95% CI = 1.00-2.13) were significantly associated with medium and high combined exposure [93]. The OR of the risk of any birth defect in the chlorination and high-color was 1.18 (95% CI = 1.02-1.36). There was no consistent association of chlorination and color with the risk of neural tube defects (OR = 1.05, 95% CI = 0.54-2.03) for combined medium and high exposure. However, the risk of neural defects for no chlorination and high-color showed a high OR of 2.60 (95% CI = 1.30-5.26). Chisholm *et al.* conducted a study to examine birth defect rates at Australia metropolitan locations with high levels of brominated DBPs [94]. Women living in high-TTHM areas (≥ 130 μg/L) showed an increased risk of any birth defect (OR = 1.22, 95% CI = 1.01-1.48) and of any cardiovascular birth defect (OR = 1.62, 95% CI = 1.04-2.51), compared with women living in low-TTHM areas (<60 μg/L). In a recent study conducted in Taiwan, the risk of ventricular septal defects (OR = 1.81, 95% CI = 0.98-3.35) and cleft palate (OR = 1.56, 95%CI = 1.00-2.41) were increased in the high TTHM (>20 μg/L) exposure group [95].

Based on the studies, it should be noted that it is still unknown whether there is a strong correlation between DBP exposure and adverse pregnancy outcomes. Several points need to be considered in advance to support a reasonable conclusion. First, the exposure assessment is a big challenge. The DBP exposure route is possible not only through ingestion, but also through showering and bathing. Therefore, a detailed questionnaire or interview is needed for better estimation of exposure, and blood sampling may improve the accuracy of actual exposure level. As shown in Table 2.2., people have tested various birth defect endpoints and different results may reflect different DBP biological impacts on specific birth defects. It is notable that the standard to categorize high- vs. low- THMs levels also varied by individual studies, and setting the level for high- and low- exposure could alter the study results. Using THM only as an

indicator of DBP levels should be reconsidered. Even though THMs are the most abundant DBP class found in drinking water, currently more than 600 DBPs have been identified and evidence demonstrated that other identified DBPs are more cytotoxic and genotoxic than THMs [96, 97]. Therefore, efforts to expand DBP chemical classes to the analysis may improve the resolving power of epidemiological studies.

2.4. TOXICITY OF COMPLEX DISINFECTION BY-PRODUCTS MIXTURES

2.4.1. Evaluation of Disinfection By-products Complex Mixtures

While investigating the toxicity of a single chemical is important, the toxicological effects of a complex DBP mixture should be considered carefully because the complex mixture represents the composition of finished water that people consume in daily life. Therefore, there is a need for toxicology research with whole DBP mixtures with extensive quantitative chemical analyses. Well-designed complex DBP mixture experiments will provide information that fills the gap between single DBP studies *in vitro* and with animal experiments to provide guidelines to estimate the health risks.

To address this issue, scientists from the U.S. EPA Office of Research and Development initiated the research project, “Integrated Disinfection Byproducts Mixtures Research: Toxicological and Chemical Evaluation of Alternative Disinfection Treatment Scenarios”, also called as the “Four Lab Study” [98]. The main objective of the Four Lab Study was to assess the reproductive/developmental effects of DBP mixtures through a “whole mixture” approach, along with chemical analyses. However, preparing complex DBP concentrate from the finished

water for toxicological researches was challenging. XAD resin extraction and elution with organic solvent is the typical method used for DBP concentrate preparation [99]. Using XAD resin extraction has limitations in the toxicological studies because the concentrate is produced in an organic solvent, which is difficult to redissolve in a water matrix for *in vivo* toxicology studies. For multigenerational rodent bioassays, large amounts of concentrates are needed, but such amounts exceed the capacity of XAD resins. Also, some volatile DBPs are often lost during the XAD resin extraction process. To resolve this issue, reverse osmosis (RO) membranes was suggested as an alternative method to concentrate large amounts of water in a short time compared to XAD, while maintaining the DBP concentrate suitable for animal studies [100]. However, the four lab study employed RO concentrate waters that contained a salt residual equivalent to about 2 g/L Na^+. These high salt levels are intrinsically toxic and may cause difficulties in the interpretation of the results. Efforts on developing new methods to concentrate DBPs in finished water are ongoing [101]. While the development of methods for complex DBP mixture preparation is actively ongoing, there are only few bioassays to investigate the *in vitro* toxicity of DBP mixtures [102]. Therefore, a good molecular metric which measures a comparative toxicity of complex mixture needs to be established.

In 2009, the European Union (EU) started a major research initiative named HIWATE (Health Impacts of long-term exposure to disinfection by-products in drinking WATEr) to address the shortcomings of previous DBP studies. The overall objective was to investigate potential human health risks including cancer and adverse reproductive outcomes associated with long-term exposure to low levels of DBPs in drinking water (www.hiwate.org) [103]. This project involved 16 teams in eight European countries and was divided into different topics

such as exposure assessment, epidemiology, risk assessment and management. For exposure assessment, DBP composition and levels in drinking water in various regions in Europe were collected where epidemiological studies are conducted. Samples were analyzed for various types of DBPs (i.e. THMs, haloacetonitirles (HANs), HAAs, haloketones (HAKs), chloral hydrate (CH), chloropicrin (CP), etc.) [104]. Therefore, the results from the HIWATE project will allow us to assess the correlation between complex DBP mixtures and human health risks.

2.4.2. Impacts of Contaminants in Source Water on Disinfection By-products Formation

Among various factors influencing the spectrum of DBPs in finished water, the composition of the source water may play an important role, especially in a local site where large amount of industrial contaminants are being discharged. Iodinated X-ray contrast media (ICM) are widely used at medical centers for soft tissues medical imaging such as organs, veins, and blood vessels. ICM are 2,4,6-triiodinated benzoic derivatives where their molecular weights varies (600 – 850 Da) by the type of side chains (Figure 2.2). The iodine atoms are responsible for the absorption of X-rays, and the compounds are designed to be persistent and polar so that the compounds can be excreted within few hours after application. After consumption, 95% of unmetabolized ICM are eliminated through urine and feces within 24 hours [105]. Incomplete removal of ICM in wastewater treatment plants could lead to an elevation of ICM concentrations in source waters such as rivers, streams, and groundwater for further disinfection by water utilities [106-108]. In a previous occurrence study involving 23 cities, four ICM including Iopamidol, Iopromide, Iohexol, and Diatrizoate were found in source waters [97]. A risk assessment on Iopromide found no toxic effects in bacteria, crustaceans, and

fish for 10 g/L Iopromide [109]. No toxic effect was found in a chronic test with *D. magna* for concentrations up to 1 g/L Iopromide as well [109].

ICM may be good sources of iodine to form iodinated DBPs, and the activated benzene rings and other functional groups that can react with oxidizing disinfectants may lead ICM to be potential DBP precursors [102]. An international collaboration study (U.S. and Germany) is being conducted to study the impacts of ICM on DBP formation and toxicity, where the overall hypothesis is that oxidizing disinfectants such as chlorine and chloramine react with ICM to form highly toxic iodo-DBPs and higher molecular weight by-products of unknown toxicity.

References

1. Xie, Y., *Disinfection Byproducts in Drinking Water; Formation, Analysis, and Control*. CRC Press/Lewis Publishers: Boca Raton, FL, 2004.
2. McGuire, M. J.; McLain, J. L.; Obolensky, A., *Information Collection Rute Data Analysis*. AwwaRF and AWWA: Denver, CO, 2002.
3. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., The occurrence of a new generation of disinfection by-products. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-7185.
4. Koudjonou, B.; Lebel, G. L.; Dabeka, L., Formation of halogenated acetaldehydes, and occurrence in Canadian drinking water. *Chemosphere* **2008,** *72*, (6), 875-881.
5. LeBel, G. L.; Benoit, F. M.; Williams, D. T., A one-year survey of halogenated disinfection by-products in the distribution system of treatment plants using three different disinfection processes. *Chemosphere* **1997,** *34*, (11), 2301-2317.
6. Williams, D. T.; LeBel, G. L.; Benoit, F. M., Disinfection by-products in Canadian drinking water. *Chemosphere* **1997,** *34*, (2), 299-316.
7. Koudjonou, B. K.; LeBel, G. L., Halogenated acetaldehydes: analysis, stability and fate in drinking water. *Chemosphere* **2006,** *64*, (5), 795-802.
8. Paode, R. D.; Amy, G. L.; Krasner, S. W.; Summers, R. S.; Rice, E. W., Predicting the formation of aldehydes and BOM. *J. Am. Water. Works. Ass.* **1997,** *89*, (6), 79-93.
9. Weinberg, H. S.; Glaze, W. H.; Krasner, S. W.; Sclimenti, M. J., Formation and Removal of Aldehydes in Plants That Use Ozonation. *J. Am. Water. Works. Ass.* **1993,** *85*, (5), 72-85.
10. Clesceri, L. S., Greenberg, A. E., Eaton, A. D. , 6252 Disinfection By-Products: Aldehydes. In *Standard Methods for the Examination of Water and Wastewater*, 20 ed.
11. EPA Method 556.1, Determination of Carbonyl Compounds in Drinking Water by Fast Gas Chromatography, Revision 1.0. In National Exposure Research Laboratory Office of Research and Development: Cincinnati, OH, 1999.
12. Guengerich, F. P.; Crawford, W. M., Jr.; Watanabe, P. G., Activation of vinyl chloride to covalently bound metabolites: roles of 2-chloroethylene oxide and 2-chloroacetaldehyde. *Biochemistry* **1979,** *18*, (23), 5177-5182.
13. Johnson, M. K., The influence of some aliphatic compounds on rat liver glutathione levels. *Biochem. Pharmacol.* **1965,** *14*, (9), 1383-1385.
14. Liviac, D.; Creus, A.; Marcos, R., DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection. *Water Res.* **2010,** *44*, (8), 2638-2646.
15. Lind, M. J.; McGown, A. T.; Hadfield, J. A.; Thatcher, N.; Crowther, D.; Fox, B. W., The effect of ifosfamide and its metabolites on intracellular glutathione levels in vitro and in vivo. *Biochem. Pharmacol.* **1989,** *38*, (11), 1835-1840.
16. Lee, C. H.; Wetmur, J. G., Physical Studies of Chloroacetaldehyde Labeled Fluorescent DNA. *Biochem. Biophys. Res. Commun.* **1973,** *50*, (3), 879-885.
17. Leonard, N. J.; Tolman, G. L., Fluorescent Nucleosides and Nucleotides. *Ann. Ny. Acad. Sci.* **1975,** *255*, (Aug8), 43-58.

18. Kochetko.Nk; Shibaev, V. N.; Kost, A. A., New Reaction of Adenine and Cytosine Derivatives, Potentially Useful for Nucleic Acids Modification. *Tetrahedron Lett.* **1971**, (22), 1993-1996.
19. Sood, C.; O'Brien, P. J., Molecular mechanisms of chloroacetaldehyde-induced cytotoxicity in isolated rat hepatocytes. *Biochem. Pharmacol.* **1993,** *46*, (9), 1621-1626.
20. Sood, C.; O'Brien, P. J., Chloroacetaldehyde-induced hepatocyte cytotoxicity. Mechanisms for cytoprotection. *Biochem. Pharmacol.* **1994,** *48*, (5), 1025-1032.
21. Benesic, A.; Schwerdt, G.; Mildenberger, S.; Freudinger, R.; Gordjani, N.; Gekle, M., Disturbed Ca2+-signaling by chloroacetaldehyde: A possible cause for chronic ifosfamide nephrotoxicity. *Kidney. Int.* **2005,** *68*, (5), 2029-2041.
22. Bolt, H. M., Roles of Etheno-DNA Adducts in Tumorigenicity of Olefins. *Crit. Rev. Toxicol.* **1988,** *18*, (4), 299-309.
23. Guengerich, F. P., Roles of the Vinyl-Chloride Oxidation-Products 2-Chlorooxirane and 2-Chloroacetaldehyde in the Invitro Formation of Etheno Adducts of Nucleic-Acid Bases. *Chem. Res. Toxicol.* **1992,** *5*, (1), 2-5.
24. Leonard, N. J., Etheno-substituted nucleotides and coenzymes: fluorescence and biological activity. *CRC Crit. Rev. Biochem.* **1984,** *15*, (2), 125-199.
25. Biernat, J.; Ciesiolka, J.; Gornicki, P.; Adamiak, R. W.; Kryzosiak, W. J.; Wiewiorowski, M., New observations concerning the chloroacetaldehyde reaction with some tRNA constituents. Stable intermediates, kinetics and selectivity of the reaction. *Nucleic Acids Res.* **1978,** *5*, (3), 789-804.
26. Basu, A. K.; Wood, M. L.; Niedernhofer, L. J.; Ramos, L. A.; Essigmann, J. M., Mutagenic and genotoxic effects of three vinyl chloride-induced DNA lesions: 1,N6-ethenoadenine, 3,N4-ethenocytosine, and 4-amino-5-(imidazol-2-yl)imidazole. *Biochemistry* **1993,** *32*, (47), 12793-12801.
27. Cheng, K. C.; Preston, B. D.; Cahill, D. S.; Dosanjh, M. K.; Singer, B.; Loeb, L. A., The vinyl chloride DNA derivative N2,3-ethenoguanine produces G----A transitions in Escherichia coli. *Proc. Natl. Acad. Sci. USA* **1991,** *88*, (22), 9974-9978.
28. Jacobsen, J. S.; Humayun, M. Z., Mechanisms of mutagenesis by the vinyl chloride metabolite chloroacetaldehyde. Effect of gene-targeted in vitro adduction of M13 DNA on DNA template activity in vivo and in vitro. *Biochemistry* **1990,** *29*, (2), 496-504.
29. Moriya, M.; Zhang, W.; Johnson, F.; Grollman, A. P., Mutagenic potency of exocyclic DNA adducts: marked differences between Escherichia coli and simian kidney cells. *Proc. Natl. Acad. Sci. USA* **1994,** *91*, (25), 11899-11903.
30. Palejwala, V. A.; Simha, D.; Humayun, M. Z., Mechanisms of mutagenesis by exocyclic DNA adducts. Transfection of M13 viral DNA bearing a site-specific adduct shows that ethenocytosine is a highly efficient RecA-independent mutagenic noninstructional lesion. *Biochemistry* **1991,** *30*, (36), 8736-8743.
31. Pandya, G. A.; Moriya, M., 1,N6-ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells. *Biochemistry* **1996,** *35*, (35), 11487-11492.
32. Singer, B.; Kusmierek, J. T.; Folkman, W.; Chavez, F.; Dosanjh, M. K., Evidence for the mutagenic potential of the vinyl chloride induced adduct, N2, 3-etheno-deoxyguanosine, using a site-directed kinetic assay. *Carcinogenesis* **1991,** *12*, (4), 745-747.

33. Singer, B.; Kusmierek, J. T.; Fraenkel-Conrat, H., In vitro discrimination of replicases acting on carcinogen-modified polynucleotide templates. *Proc. Natl. Acad. Sci. USA* **1983,** *80*, (4), 969-972.
34. Barbin, A.; Bartsch, H.; Leconte, P.; Radman, M., Studies on the miscoding properties of 1,N6-ethenoadenine and 3,N4-ethenocytosine, DNA reaction products of vinyl chloride metabolites, during in vitro DNA synthesis. *Nucleic Acids Res.* **1981,** *9*, (2), 375-387.
35. Choi, J. H.; Pfeifer, G. P., DNA damage and mutations produced by chloroacetaldehyde in a CpG-methylated target gene. *Mutat. Res.* **2004,** *568*, (2), 245-256.
36. Malaveille, C.; Bartsch, H.; Barbin, A.; Camus, A. M.; Montesano, R.; Croisy, A.; Jacquignon, P., Mutagenicity of Vinyl-Chloride, Chloroethyleneoxide, Chloroacetaldehyde and Chloroethanol. *Biochem. Bioph. Res. Co.* **1975,** *63*, (2), 363-370.
37. Mccann, J.; Simmon, V.; Streitwieser, D.; Ames, B. N., Mutagenicity of Chloracetaldehyde, a Possible Metabolic Product of 1,2-Dichloroethane (Ethylene Dichloride), Chloroethanol (Ethylene Chlorohydrin), Vinyl-Chloride, and Cyclophosphamide. *Proc. Natl. Acad. Sci. USA* **1975,** *72*, (8), 3190-3193.
38. Guengerich, F. P.; Mason, P. S.; Stott, W. T.; Fox, T. R.; Watanabe, P. G., Roles of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res.* **1981,** *41*, (11 Pt 1), 4391-4398.
39. Huberman, E.; Bartsch, H.; Sachs, L., Mutation Induction in Chinese-Hamster V79 Cells by 2 Vinyl-Chloride Metabolites, Chloroethylene Oxide and 2-Chloroacetaldehyde. *Int. J. Cancer* **1975,** *16*, (4), 639-644.
40. Crebelli, R.; Conti, G.; Conti, L.; Carere, A., Chloroacetaldehyde Is a Powerful Inducer of Mitotic Aneuploidy in Aspergillus-Nidulans. *Mutagenesis* **1990,** *5*, (2), 165-168.
41. Maciejewska, A. M.; Ruszel, K. P.; Nieminuszczy, J.; Lewicka, J.; Sokolowska, B.; Grzesiuk, E.; Kusmierek, J. T., Chloroacetaldehyde-induced mutagenesis in Escherichia coli: the role of AlkB protein in repair of 3,N(4)-ethenocytosine and 3,N(4)-alpha-hydroxyethanocytosine. *Mutat. Res.* **2010,** *684*, (1-2), 24-34.
42. Spengler, S. J.; Singer, B., Formation of interstrand cross-links in chloroacetaldehyde-treated DNA demonstrated by ethidium bromide fluorescence. *Cancer Res.* **1988,** *48*, (17), 4804-4806.
43. Daniel, F. B.; DeAngelo, A. B.; Stober, J. A.; Olson, G. R.; Page, N. P., Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. *Fundam. Appl. Toxicol.* **1992,** *19*, (2), 159-168.
44. Goldenthal, E. I., A compilation of LD50 values in newborn and adult animals. *Toxicol. Appl. Pharmacol.* **1971,** *18*, (1), 185-207.
45. Sanders, V. M.; Kauffmann, B. M.; White, K. L., Jr.; Douglas, K. A.; Barnes, D. W.; Sain, L. E.; Bradshaw, T. J.; Borzelleca, J. F.; Munson, A. E., Toxicology of chloral hydrate in the mouse. *Environ. Health Perspect.* **1982,** *44*, 137-146.
46. Beland, F. A., NTP technical report on the toxicity and metabolism studies of chloral hydrate (CAS No. 302-17-0). Administered by gavage to F344/N rats and B6C3F1 mice. *Toxic. Rep. Ser.* **1999**, (59), 1-66, A1-E7.
47. Haworth, S.; Lawlor, T.; Mortelmans, K.; Speck, W.; Zeiger, E., Salmonella mutagenicity test results for 250 chemicals. *Environ. Mutagen.* **1983,** *5 Suppl 1*, 1-142.

48. Leuschner, J.; Leuschner, F., Evaluation of the mutagenicity of chloral hydrate in vitro and in vivo. *Arzneim.-Forsch.* **1991,** *41*, (10), 1101-1103.
49. Ni, Y. C.; Wong, T. Y.; Kadlubar, F. F.; Fu, P. P., Hepatic metabolism of chloral hydrate to free radical(s) and induction of lipid peroxidation. *Biochem. Biophys. Res. Commun.* **1994,** *204*, (2), 937-943.
50. Furnus, C. C.; Ulrich, M. A.; Terreros, M. C.; Dulout, F. N., The induction of aneuploidy in cultured Chinese hamster cells by propionaldehyde and chloral hydrate. *Mutagenesis* **1990,** *5*, (4), 323-326.
51. Mailhes, J. B.; Preston, R. J.; Yuan, Z. P.; Payne, H. S., Analysis of mouse metaphase II oocytes as an assay for chemically induced aneuploidy. *Mutat. Res.* **1988,** *198*, (1), 145-152.
52. Russo, A.; Pacchierotti, F.; Metalli, P., Nondisjunction induced in mouse spermatogenesis by chloral hydrate, a metabolite of trichloroethylene. *Environ. Mutagen.* **1984,** *6*, (5), 695-703.
53. Allen, J. W.; Collins, B. W.; Evansky, P. A., Spermatid micronucleus analyses of trichloroethylene and chloral hydrate effects in mice. *Mutat. Res.* **1994,** *323*, (1-2), 81-88.
54. Nesslany, F.; Marzin, D., A micromethod for the in vitro micronucleus assay. *Mutagenesis* **1999,** *14*, (4), 403-410.
55. Parry, J. M.; Parry, E. M.; Bourner, R.; Doherty, A.; Ellard, S.; O'Donovan, J.; Hoebee, B.; de Stoppelaar, J. M.; Mohn, G. R.; Onfelt, A.; Renglin, A.; Schultz, N.; Soderpalm-Berndes, C.; Jensen, K. G.; Kirsch-Volders, M.; Elhajouji, A.; Van Hummelen, P.; Degrassi, F.; Antoccia, A.; Cimini, D.; Izzo, M.; Tanzarella, C.; Adler, I. D.; Kliesch, U.; Hess, P.; et al., The detection and evaluation of aneugenic chemicals. *Mutat. Res.* **1996,** *353*, (1-2), 11-46.
56. Parry, J. M.; Parry, E. M.; Warr, T.; Lynch, A.; James, S., The detection of aneugens using yeasts and cultured mammalian cells. *Prog. Clin. Biol. Res.* **1990,** *340B*, 247-266.
57. Migliore, L.; Nieri, M., Evaluation of twelve potential aneuploidogenic chemicals by the in vitro human lymphocyte micronucleus assay. *Toxicol. In Vitro* **1991,** *5*, (4), 325-336.
58. Brunner, M.; Albertini, S.; Wurgler, F. E., Effects of 10 known or suspected spindle poisons in the in vitro porcine brain tubulin assembly assay. *Mutagenesis* **1991,** *6*, (1), 65-70.
59. Eichenlaub-Ritter, U.; Baart, E.; Yin, H.; Betzendahl, I., Mechanisms of spontaneous and chemically-induced aneuploidy in mammalian oogenesis: basis of sex-specific differences in response to aneugens and the necessity for further tests. *Mutat. Res.* **1996,** *372*, (2), 279-294.
60. Warr, T. J.; Parry, E. M.; Parry, J. M., A comparison of two in vitro mammalian cell cytogenetic assays for the detection of mitotic aneuploidy using 10 known or suspected aneugens. *Mutat. Res.* **1993,** *287*, (1), 29-46.
61. Moore, M. M.; Harrington-Brock, K., Mutagenicity of trichloroethylene and its metabolites: implications for the risk assessment of trichloroethylene. *Environ. Health Perspect.* **2000,** *108 Suppl 2*, 215-223.
62. Nelson, M. A.; Bull, R. J., Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver in vivo. *Toxicol. Appl. Pharmacol.* **1988,** *94*, (1), 45-54.

63. Bernstine, J. B.; Meyer, A. E.; Hayman, H. B., Maternal and foetal blood estimation following the administration of chloral hydrate during labour. *J. Obstet. Gynaecol. Br. Emp.* **1954,** *61*, (5), 683-685.
64. Lambert, G. H.; Muraskas, J.; Anderson, C. L.; Myers, T. F., Direct hyperbilirubinemia associated with chloral hydrate administration in the newborn. *Pediatrics* **1990,** *86*, (2), 277-281.
65. Eichenlaub-Ritter, U.; Betzendahl, I., Chloral hydrate induced spindle aberrations, metaphase I arrest and aneuploidy in mouse oocytes. *Mutagenesis* **1995,** *10*, (6), 477-486.
66. Klinefelter, G. R.; Suarez, J. D.; Roberts, N. L.; DeAngelo, A. B., Preliminary screening for the potential of drinking water disinfection byproducts to alter male reproduction. *Reprod. Toxicol.* **1995,** *9*, (6), 571-578.
67. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 disinfectants and disinfection byproducts rule. *Fed. Reg.* **2006,** *71*, (2), 387-493.
68. Weinberg, H. S.; Krasner, S. W.; Richardson, S. D.; Thruston, A. D., Jr. *The Occurrence of Disinfection By-Products (DBPs) of Health Concern in Drinking Water: Results of a Nationwide DBP Occurrence Study*; EPA/600/R02/068; U.S. Environmental Protection Agency National Exposure Research Laboratory: Athens, GA., 2002; pp 1-460.
69. Cemeli, E.; Wagner, E. D.; Anderson, D.; Richardson, S. D.; Plewa, M. J., Modulation of the cytotoxicity and genotoxicity of the drinking water disinfection byproduct Iodoacetic acid by suppressors of oxidative stress. *Environ. Sci. Technol.* **2006,** *40*, (6), 1878-1883.
70. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in *Salmonella typhimurium*. *Teratog. Carcinog. Mutagen.* **2002,** *22*, (2), 113-128.
71. Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2010,** *51*, (8-9), 871-878.
72. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D., Jr.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38*, (18), 4713-4722.
73. Zhang, S. H.; Miao, D. Y.; Liu, A. L.; Zhang, L.; Wei, W.; Xie, H.; Lu, W. Q., Assessment of the cytotoxicity and genotoxicity of haloacetic acids using microplate-based cytotoxicity test and CHO/HGPRT gene mutation assay. *Mutat. Res.* **2010,** *703*, (2), 174-179.
74. Hilliard, C. A.; Armstrong, M. J.; Bradt, C. I.; Hill, R. B.; Greenwood, S. K.; Galloway, S. M., Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. *Environ. Mol. Mutagen.* **1998,** *31*, (4), 316-326.
75. Fuscoe, J. C.; Afshari, A. J.; George, M. H.; DeAngelo, A. B.; Tice, R. R.; Salman, T.; Allen, J. W., In vivo genotoxicity of dichloroacetic acid: evaluation with the mouse peripheral blood micronucleus assay and the single cell gel assay. *Environ. Mol. Mutagen.* **1996,** *27*, (1), 1-9.
76. Harrington-Brock, K.; Doerr, C. L.; Moore, M. M., Mutagenicity of three disinfection by-products: di- and trichloroacetic acid and chloral hydrate in L5178Y/TK +/- (-)3.7.2C mouse lymphoma cells. *Mutat. Res.* **1998,** *413*, (3), 265-276.

77. Hunter, E. S.; Rogers, E. H.; Schmid, J. E.; Richard, A., Comparative effects of haloacetic acids in whole embryo culture. *Teratology* **1996,** *54*, (2), 57-64.
78. Richard, A. M.; Hunter, E. S., 3rd, Quantitative structure-activity relationships for the developmental toxicity of haloacetic acids in mammalian whole embryo culture. *Teratology* **1996,** *53*, (6), 352-360.
79. Wei, X.; Wang, S.; Zheng, W.; Wang, X.; Liu, X.; Jiang, S.; He, G.; Zheng, Y.; Qu, W., Tumorigenicity of drinking water disinfection byproduct iodoacetic acid in NIH3T3 cells. *Envion. Sci. Technol.* **2013,** *47*, (11), 5913-5920.
80. Narotsky, M. G.; Best, D. S.; McDonald, A.; Godin, E. A.; Hunter, E. S., 3rd; Simmons, J. E., Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. *Reprod. Toxicol.* **2011,** *31*, (1), 59-65.
81. George, S. E.; Nelson, G. M.; Swank, A. E.; Brooks, L. R.; Bailey, K.; George, M.; DeAngelo, A., The disinfection by-products dichloro-, dibromo-, and bromochloroacetic acid impact intestinal microflora and metabolism in Fischer 344 rats upon exposure in drinking water. *Toxicol. Sci.* **2000,** *56*, (2), 282-289.
82. Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44*, (19), 7206-7212.
83. Muellner, M. G.; Attene-Ramos, M. S.; Hudson, M. E.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis of bromoacetic acid: a regulated drinking water disinfection by-product. *Environ. Mol. Mutagen.* **2010,** *51*, 205-214.
84. Hernandez-Fonseca, K.; Cardenas-Rodriguez, N.; Pedraza-Chaverri, J.; Massieu, L., Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons. *J. Neurosci. Res.* **2008,** *86*, (8), 1768-1780.
85. Kahlert, S.; Reiser, G., Requirement of glycolytic and mitochondrial energy supply for loading of $Ca^{(2+)}$ stores and InsP(3)-mediated $Ca^{(2+)}$ signaling in rat hippocampus astrocytes. *J. Neurosci. Res.* **2000,** *61*, (4), 409-420.
86. Pals, J. A.; Ang, J. K.; Wagner, E. D.; Plewa, M. J., Biological mechanism for the toxicity of haloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2011,** *45*, (13), 5791-5797.
87. Mills, C. J.; Bull, R. J.; Cantor, K. P.; Reif, J. S.; Hrudey, S. E.; Huston, P. *Health risks of drinking water chlorination by-products: report of an exper working group*; Chronic Diseases in Canada: 1998; pp 91-102.
88. Cantor, K. P.; Lynch, C. F.; Hildesheim, M. E.; Dosemeci, M.; Lubin, J.; Alavanja, M.; Craun, G., Drinking water source and chlorination byproducts I. Risk of bladder cancer. *Epidemiology* **1998,** *9*, (1), 21-28.
89. Doyle, T. J.; Zheng, W.; Cerhan, J. R.; Hong, C. P.; Sellers, T. A.; Kushi, L. H.; Folsom, A. R., The association of drinking water source and chlorination by-products with cancer incidence among postmenopausal women in Iowa: a prospective cohort study. *Am. J. Public Health* **1997,** *87*, (7), 1168-1176.

90. Freedman, D. M.; Cantor, K. P.; Lee, N. L.; Chen, L. S.; Lei, H. H.; Ruhl, C. E.; Wang, S. S., Bladder cancer and drinking water: a population-based case-control study in Washington County, Maryland (United States). *Cancer Causes Control* **1997,** *8*, (5), 738-744.
91. King, W. D.; Marrett, L. D.; Woolcott, C. G., Case-control study of colon and rectal cancers and chlorination by-products in treated water. *Cancer Epidemiol. Biomarkers Prev.* **2000,** *9*, (8), 813-818.
92. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39*, (3), 733-745.
93. Hwang, B. F.; Magnus, P.; Jaakkola, J. J., Risk of specific birth defects in relation to chlorination and the amount of natural organic matter in the water supply. *Am. J. Epidemiol.* **2002,** *156*, (4), 374-382.
94. Chisholm, K.; Cook, A.; Bower, C.; Weinstein, P., Risk of birth defects in Australian communities with high levels of brominated disinfection by-products. *Environ. Health Perspect.* **2008,** *116*, (9), 1267-1273.
95. Hwang, B. F.; Jaakkola, J. J.; Guo, H. R., Water disinfection by-products and the risk of specific birth defects: a population-based cross-sectional study in Taiwan. *Environ. Health* **2008,** *7*, 23.
96. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
97. Richardson, S. D.; Fasano, F.; Ellington, J. J.; Crumley, F. G.; Buettner, K. M.; Evans, J. J.; Blount, B. C.; Silva, L. K.; Waite, T. J.; Luther, G. W.; McKague, A. B.; Miltner, R. J.; Wagner, E. D.; Plewa, M. J., Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. *Environ. Sci. Technol.* **2008,** *42*, (22), 8330-8338.
98. Simmons, J. E.; Richardson, S. D.; Teuschler, L. K.; Miltner, R. J.; Speth, T. F.; Schenck, K. M.; Hunter, E. S., 3rd; Rice, G., Research issues underlying the four-lab study: integrated disinfection by-products mixtures research. *J. Toxicol. Environ. Health A* **2008,** *71*, (17), 1125-1132.
99. Simmons, J. E.; Richardson, S. D.; Speth, T. F.; Miltner, R. J.; Rice, G.; Schenck, K. M.; Hunter, E. S., 3rd; Teuschler, L. K., Development of a research strategy for integrated technology-based toxicological and chemical evaluation of complex mixtures of drinking water disinfection byproducts. *Environ. Health Perspect.* **2002,** *110 Suppl 6*, 1013-1024.
100. Speth, T. F.; Miltner, R. J.; Richardson, S. D.; Simmons, J. E., Integrated disinfection by-products mixtures research: concentration by reverse osmosis membrane techniques of disinfection by-products from water disinfected by chlorination and ozonation/postchlorination. *J. Toxicol. Environ. Health A* **2008,** *71*, (17), 1149-1164.
101. Pressman, J. G.; Richardson, S. D.; Speth, T. F.; Miltner, R. J.; Narotsky, M. G.; Hunter, E. S., 3rd; Rice, G. E.; Teuschler, L. K.; McDonald, A.; Parvez, S.; Krasner, S. W.; Weinberg, H. S.; McKague, A. B.; Parrett, C. J.; Bodin, N.; Chinn, R.; Lee, C. F.; Simmons, J. E., Concentration, chlorination, and chemical analysis of drinking water for disinfection byproduct mixtures health effects research: U.S. EPA's Four Lab Study. *Environ. Sci. Technol.* **2010,** *44*, (19), 7184-7192.

102. Duirk, S. E.; Lindell, C.; Cornelison, C. C.; Kormos, J.; Ternes, T. A.; Attene-Ramos, M.; Osiol, J.; Wagner, E. D.; Plewa, M. J.; Richardson, S. D., Formation of toxic iodinated disinfection by-products from compounds used in medical imaging. *Environ. Sci. Technol.* **2011,** *45*, (16), 6845-6854.
103. Nieuwenhuijsen, M. J.; Smith, R.; Golfinopoulos, S.; Best, N.; Bennett, J.; Aggazzotti, G.; Righi, E.; Fantuzzi, G.; Bucchini, L.; Cordier, S.; Villanueva, C. M.; Moreno, V.; La Vecchia, C.; Bosetti, C.; Vartiainen, T.; Rautiu, R.; Toledano, M.; Iszatt, N.; Grazuleviciene, R.; Kogevinas, M., Health impacts of long-term exposure to disinfection by-products in drinking water in Europe: HIWATE. *J. Water Health* **2009,** *7*, (2), 185-207.
104. Goslan, E. H.; Krasner, S. W.; Bower, M.; Rocks, S. A.; Holmes, P.; Levy, L. S.; Parsons, S. A., A comparison of disinfection by-products found in chlorinated and chloraminated drinking waters in Scotland. *Water Res.* **2009,** *43*, (18), 4698-4706.
105. Perez, S.; Eichhorn, P.; Celiz, M. D.; Aga, D. S., Structural characterization of metabolites of the X-ray contrast agent iopromide in activated sludge using ion trap mass spectrometry. *Anal. Chem.* **2006,** *78*, (6), 1866-1874.
106. Carballa, M.; Omil, F.; Lema, J. M.; Llompart, M.; Garcia-Jares, C.; Rodriguez, I.; Gomez, M.; Ternes, T., Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* **2004,** *38*, (12), 2918-2926.
107. Drewes, J. E.; Fox, P.; Jekei, M., Occurrence of iodinated x-ray contrast media in domestic effluents and their fate during indirect potable reuse. *J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng.* **2001,** *36*, (9), 1633-1645.
108. Sacher, F.; Lange, F. T.; Brauch, H. J.; Blankenhorn, I., Pharmaceuticals in groundwaters analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *J. Chromatogr. A* **2001,** *938*, (1-2), 199-210.
109. Steger-Hartmann, T.; Lange, R.; Schweinfurth, H., Environmental risk assessment for the widely used iodinated X-ray contrast agent iopromide (Ultravist). *Ecotoxicol. Environ. Saf.* **1999,** *42*, (3), 274-281.
110. Wilkins, J. R., 3rd; Comstock, G. W., Source of drinking water at home and site-specific cancer incidence in Washington County, Maryland. *Am. J. Epidemiol.* **1981,** *114*, (2), 178-190.
111. Cantor, K. P.; Hoover, R.; Hartge, P.; Mason, T. J.; Silverman, D. T.; Altman, R.; Austin, D. F.; Child, M. A.; Key, C. R.; Marrett, L. D.; et al., Bladder cancer, drinking water source, and tap water consumption: a case-control study. *J. Natl. Cancer Inst.* **1987,** *79*, (6), 1269-1279.
112. Lynch, C. F.; Woolson, R. F.; Ogorman, T.; Cantor, K. P., Chlorinated Drinking-Water and Bladder-Cancer - Effect of Misclassification on Risk Estimates. *Arch Environ Health* **1989**, 44, (4), 252-259.
113. Cordier, S.; Clavel, J.; Limasset, J. C.; Boccongibod, L.; Lemoual, N.; Mandereau, L.; Hemon, D., Occupational Risks of Bladder-Cancer in France - a Multicenter Case-Control Study. *Int. J. Epidemiol.* **1993**, 22, (3), 403-411.
114. McGeehin, M. A.; Reif, J. S.; Becher, J. C.; Mangione, E. J., Case-control study of bladder cancer and water disinfection methods in Colorado. *Am. J. Epidemiol.* **1993,** *138*, (7), 492-501.

115. King, W. D.; Marrett, L. D., Case-control study of bladder cancer and chlorination by-products in treated water (Ontario, Canada). *Cancer Causes Control* **1996,** *7*, (6), 596-604.
116. Koivusalo, M.; Hakulinen, T.; Vartiainen, T.; Pukkala, E.; Jaakkola, J. J.; Tuomisto, J., Drinking water mutagenicity and urinary tract cancers: a population-based case-control study in Finland. *Am. J. Epidemiol.* **1998,** *148*, (7), 704-712.
117. Chevrier, C.; Junod, B.; Cordier, S., Does ozonation of drinking water reduce the risk of bladder cancer? *Epidemiology* **2004,** *15*, (5), 605-614.
118. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.
119. Bove, G. E., Jr.; Rogerson, P. A.; Vena, J. E., Case-control study of the effects of trihalomethanes on urinary bladder cancer risk. *Arch. Environ. Occup. Health* **2007,** *62*, (1), 39-47.
120. Michaud, D. S.; Kogevinas, M.; Cantor, K. P.; Villanueva, C. M.; Garcia-Closas, M.; Rothman, N.; Malats, N.; Real, F. X.; Serra, C.; Garcia-Closas, R.; Tardon, A.; Carrato, A.; Dosemeci, M.; Silverman, D. T., Total fluid and water consumption and the joint effect of exposure to disinfection by-products on risk of bladder cancer. *Environ. Health Perspect.* **2007,** *115*, (11), 1569-1572.
121. Aschengrau, A.; Zierler, S.; Cohen, A., Quality of community drinking water and the occurrence of late adverse pregnancy outcomes. *Archives of Environmental Health* **1993,** *48*, (2), 105-113.
122. Bove, F. J.; Fulcomer, M. C.; Klotz, J. B.; Esmart, J.; Dufficy, E. M.; Savrin, J. E., Public drinking water contamination and birth outcomes. *Am. J. Epidemiol.* **1995,** *141*, (9), 850-862.
123. Magnus, P.; Jaakkola, J. J.; Skrondal, A.; Alexander, J.; Becher, G.; Krogh, T.; Dybing, E., Water chlorination and birth defects. *Epidemiology* **1999,** *10*, (5), 513-517.
124. Wright, J. M.; Schwartz, J.; Dockery, D. W., Effect of trihalomethane exposure on fetal development. *Occup. Environ. Med.* **2003**, 60, (3), 173-180.
125. Hinckley, A. F.; Bachand, A. M.; Reif, J. S., Late pregnancy exposures to disinfection by-products and growth-related birth outcomes. *Environ. Health Perspect.* **2005**, 113, (12), 1808-1813.
126. Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ. Res.* **2007**, 104, (3), 390-395.

TABLES AND FIGURES

Table 2.1. Summary of epidemiology studies on urinary bladder cancer associated with chlorinated DBPs exposure.

Exposure Comparison	Adjusted OR (95% CI)	Study Design (Country)	Reference (year)
Male: Municipal (disinfected) water[a] Female: Municipal (disinfected) water[a]	1.80 (0.80-4.75) 1.60 (0.54-6.32)	Nonconcurrent prospective (MD, U.S.)	Wilkins & Comstock (1981) [110]
Male: 40-59 yrs, chlorinated surface water[b] Male: ≥60 yrs, chlorinated surface water[b] Female: ≥ 60 yrs, chlorinated surface water[b]	1.2 (0.8-1.7) 1.2 (0.7-2.1) 3.2 (1.2-8.7)*	Case-control (IA, U.S.)	Cantor *et al.* (1987) [111]
Male: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c] Female: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c] Both: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c]	1.37 (0.95-1.98) 1.97 (0.92-4.21) 1.52 (1.10-2.10)	Case-control (IA, U.S.)	Lynch *et al.* (1989) [112]
Male: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c] Female: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c] Both: ≥40 yrs, 1 µg/L THM vs. ≤ 1 µg/L THM[c]	1.20 (0.74-1.94) 0.46 (0.14-1.55) 1.02 (0.66-1.57)	Case-control (France)	Cordier *et al.* (1993) [113]
Both: 21-30 yrs, any chlorinated water[d] Both: >30 yrs, any chlorinated water[d]	1.5 (0.8-2.9) 1.8 (1.1-2.9)*	Case-control (CO, U.S.)	McGeehin *et al.* (1993) [114]
Both: >35 yrs, chlorinated surface water[d] Both: >35 yrs, ≥25 µg/L TTHM surface water[d]	1.41 (1.09-1.81)* 1.58 (1.17-2.14)*	Case-control (Canada)	King & Marrett (1996) [115]
Male: >40 yrs, Municipal (disinfected) water[e] Female: >40 yrs, Municipal (disinfected) water[e]	2.2 (0.8-5.1) 0.6 (0.2-2.2)	Case-control (MD, U.S.)	Freedman *et al.* (1997) [90]
Female: 25 µg/L TTHM vs. < limit of detection	0.62 (0.25-1.59)	Cohort (IA, U.S.)	Doyle *et al.* (1997) [89]
Male: 40-59 yrs, chlorinated surface water[b] Male: ≥60 yrs, chlorinated surface water[b] Female: ≥ 60 yrs, chlorinated surface water[b]	1.5 (0.95-2.3) 1.9 (1.1-3.6)* 0.7 (0.2-2.4)	Case-control (IA, U.S.)	Cantor *et al.* (1998) [88]
Both: >30 yrs, mutagenic chlorinated water[f]	1.22 (0.92-1.62)	Case-control (Finland)	Koivusalo *et al.* (1998) [116]
Both: >50 µg/L TTHM vs. <1 µg/L TTHM	2.99 (1.1-8.5)*	Case-control (France)	Chevrier *et al.* (2004) [117]
Male: >25-30 yrs, chlorinated surface water[d] Male: >30 yrs, chlorinated surface water[d] Female: >30 yrs, chlorinated surface water[d]	2.58 (1.33-5.01)* 2.21 (1.17-4.20)* 2.33 (0.51-10.55)	Case-control (Spain)	Villanueva *et al.* (2007) [118]
Both: 74-352 µg/L TTHM vs. 0-38 µg/L TTHM	2.34 (1.01-3.66)*	Case-control (NY, U.S.)	Bove *et al.* (2007) [119]

Table 2.1. (Continued)

Exposure Comparison	Adjusted OR (95% CI)	Study Design (Country)	Reference (year)
Both: 26-49 µg/L TTHM vs. ≤ 8 µg/L TTHM Both: >49 µg/L TTHM vs. ≤ 8 µg/L TTHM	2.34 (1.16-4.71)* 2.06 (0.83-5.08)	Case-control (Spain)	Michaud *et al.* (2007) [120]

Abbreviations; TTHM = total THM. * Statistically significant. [a] Compared with deep well water users. [b] Compared with unchlorinated water or chlorinated groundwater. [c] Data represented by Villanueva *et al.* [19]. [d] Compared with unchlorinated water. [e] Compared with non-municipal water. [f] Compared with non-mutagenic water.

Table 2.2. Summary of epidemiology studies on adverse pregnancy outcomes associated with chlorinated DBPs exposure.

Endpoint and Exposure Comparison	Adjusted OR (95% CI)	Study Design (Country)	Reference (year)
Stillbirth: public water supplies from the communities (chlorinated surface water)	2.6 (0.9-7.5)	Case-control (MA, U.S.)	Aschengrau *et al.* (1993) [121]
Low birth weight: TTHM >100 ppb vs. <20 ppb Small for gestational age: TTHM >100 ppb vs. <20 ppb All surveillance birth defects: TTHM >100 ppb vs. <20 ppb All surveillance birth defects: TTHM >80 ppb vs. <20 ppb	 1.42 (1.22-1.65)[a] 1.50 (1.36-1.65)[a] 1.04 (0.58-1.76)[b] 1.57 (1.23-1.99)[b]	Cross-sectional (NJ, U.S.)	Bove *et al.* (1995) [122]
Any malformation: Colored chlorinated water vs. no chlorination Neural tube defects: Colored chlorinated water vs. no chlorination Urinary tract defects: Colored chlorinated water vs. no chlorination	 1.14 (0.99-1.31) 1.26 (0.61-2.62) 1.99 (1.10-33.57)	Retrospective (Norway)	Magnus *et al.* (1999) [123]
Any birth defect: Chlorination and high-color Neural tube defects: No chlorination and high-color	1.18 (1.02-1.36) 1.05 (0.54-2.03)	Cross-sectional (Norway)	Hwang *et al.* (2002) [93]
Small for gestational age(pregnancy average): TTHM levels >80 µg/L vs. ≤ 60 µg/L Small for gestational age(second trimester): TTHM levels >80 µg/L vs. ≤ 60 µg/L	1.14 (1.02-1.26) 1.13 (1.03-1.24)	Cross-sectional (MA, U.S.)	Wright *et al.* (2003) [124]
Term low birth weight: HAA5 <15 µg/L vs. HAA5 15-19 µg/L HAA5 <15 µg/L vs. HAA5 ≥19 µg/L	 1.26 (0.96-1.65) 1.25 (0.96-1.64)	Retrospective (AZ, U.S.)	Hinckley *et al.* (2005) [125]
Preterm delivery: TTHM <4.93 µg/L vs. TTHM 4.93–13.11 µg/L TTHM <4.93 µg/L vs. TTHM >13.11 µg/L	 1.03 (0.94–1.13) 1.08 (0.98–1.18)	Retrospective (Taiwan)	Yang et al. (2007) [126]

Table 2.2. (Continued)

Endpoint and Exposure Comparison	Adjusted OR (95% CI)	Study Design (Country)	Reference (year)
Any birth defect: High-TTHM (≥ 130 µg/L) vs. low (<60 µg/L) Any cardiovascular birth defect: High-TTHM (≥ 130 µg/L) vs. low (<60 µg/L)	1.22 (1.01-1.48) 1.62 (1.04-2.51)	Prevalence design (Australia)	Chisholm *et al.* (2008) [94]
Ventricular septal defects: High-TTHM (>20 µg/L) vs. reference (0-4 µg/L) Cleft palate: High-TTHM (>20 µg/L) vs. reference (0-4 µg/L)	1.81 (0.98-3.35) 1.56 (1.00-2.41)	Cross-sectional (Taiwan)	Hwang *et al.* (2008) [95]

Abbreviations; TTHM = total THM. * Statistically significant. [a] 50% confidence interval. . [b] 90% confidence interval.

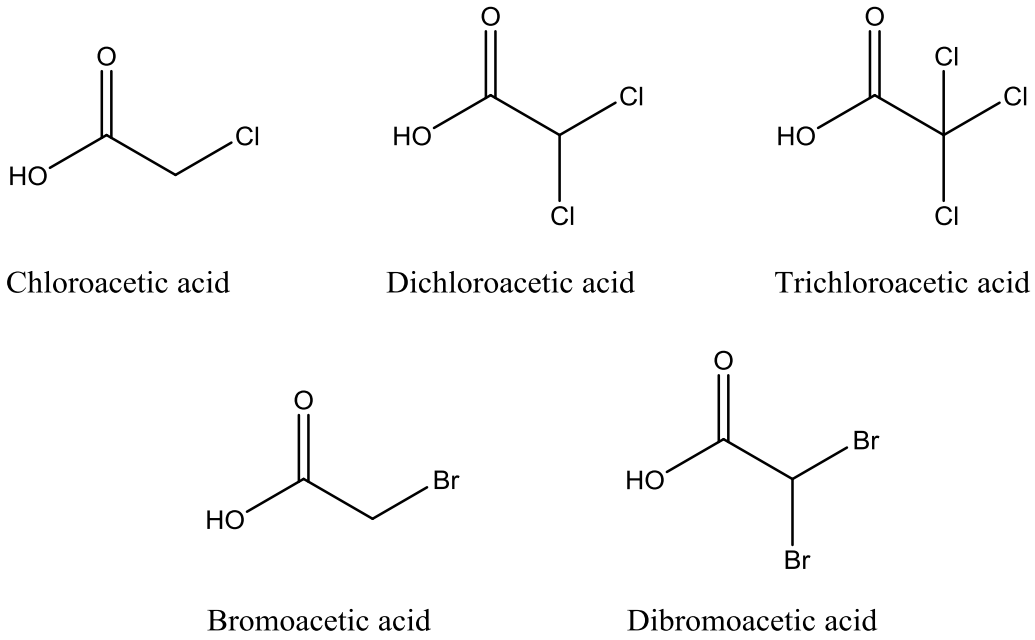
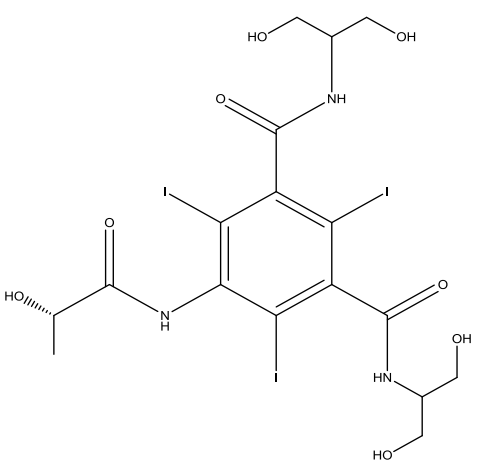
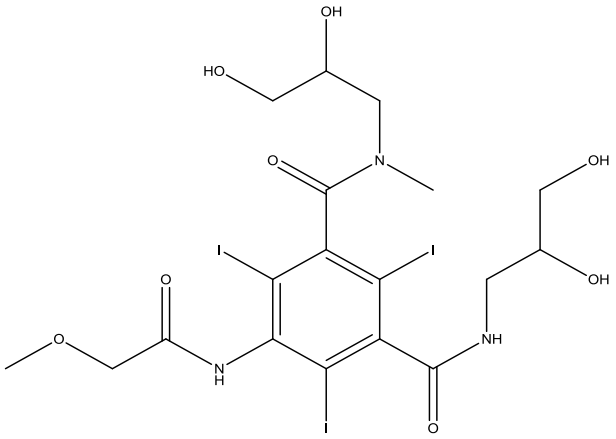


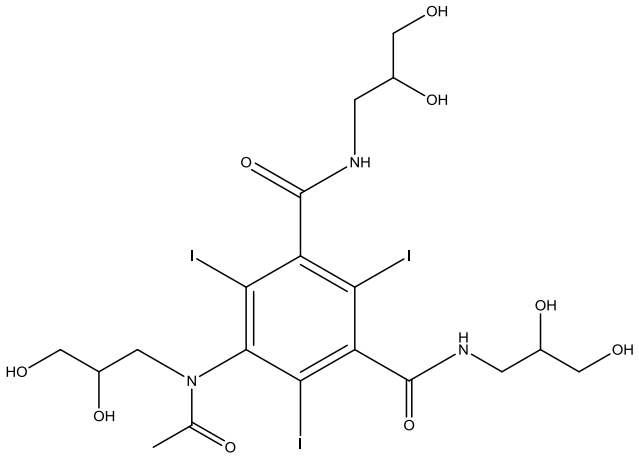
Figure 2.1. Chemical structures of regulated five haloacetic acid DBPs



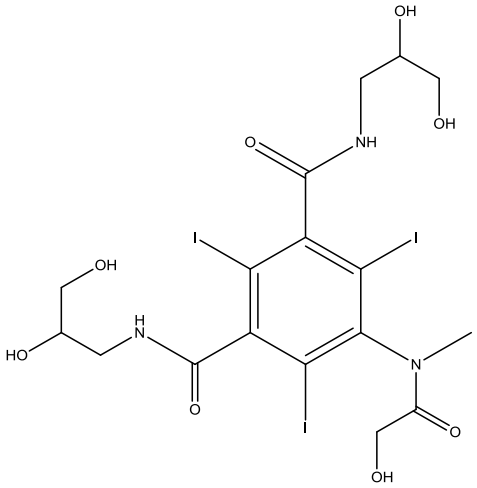
Iopamidol
CAS No. 62883-00-5
776.85 Da



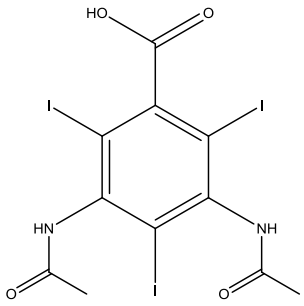
Iopromide
CAS No. 73334-07-3
790.87 Da



Iohexol
CAS No. 66108-95-0
820.88 Da



Iomeprol
CAS No. 78649-41-9
776.85 Da



Diatrizoate
CAS No. 737-31-5
613.77 Da

Figure 2.2. Chemical structures of iodinated X-ray contrast agents.

CHAPTER 3

OCCURRENCE AND COMPARATIVE TOXICITY OF THE HALOACETALDEHYDE DISINFECTION BY-PRODUCTS IN DRINKING WATER

PREFACE

This work was a collaboration study involving research on analytical biology and analytical chemistry. Dr. C. Postigo (IDAEA-CSIC, Barcelona, Spain) conducted the analytical chemistry component under the direction of Dr. S. Richardson (University of South Carolina, SC).

3.1. INTRODUCTION

The disinfection of drinking water was an outstanding contribution for the protection of the public health [1]. An unintended consequence of water disinfection is the generation of disinfection by-products (DBPs). Since the trihalomethanes (THMs) were discovered as the first chemical class of DBPs [2], research lead to the identification of emerging DBPs [3-6], formation kinetics [7-9], exposure assessment [10-13], risk assessment [12, 14], and toxicology [15-17]. Epidemiological studies demonstrated associations between DBPs with increased risk for bladder, stomach, and colon cancers [18-22]. Evidence was found associating DBPs and adverse pregnancy outcomes, including spontaneous abortion, low birth weight, small-for-gestational-age (SGA), still birth, and preterm delivery [11, 23-29]. Many DBPs are cytotoxic, genotoxic,

teratogenic and carcinogenic [15, 17, 30-33]. To date, more than 600 DBPs have been identified with the haloacetaldehydes (HALs) as one of the emerging DBP chemical classes [34].

Aldehyde DBPs are preferentially formed with ozone disinfection; levels of halogenated and non-halogenated aldehydes range from 5 to 20 μg/L [35]. In the U.S. EPA Information Collection Rule (ICR), HALs were found at higher concentrations in water treatment systems using ozone than with chlorine dioxide (up to 30.6 μg/L) [36]. Chloral hydrate (trichloroacetaldehyde, TCAL) was the major HAL; chloroacetaldehyde (CAL) and dichloroacetaldehyde (DCAL) were also formed in disinfected water. However, the evaluation of the occurrence of individual HALs is limited because some can be transformed into TCAL in water [35]. Brominated HALs such as bromochloroacetaldehyde (BCAL), dibromoacetaldehyde (DBAL), bromodichloroacetaldehyde (BDCAL), dibromochloroacetaldehyde (DBCAL) and tribromoacetaldehyde (TBAL) were detected when bromide-containing waters were chlorinated [37]. In a nationwide occurrence study, 4 HALs (CAL, DCAL, BCAL, and TCAL) were included as priority DBPs. HALs were the third largest DBP class by weight, with DCAL as the most abundant HAL detected (maximum concentration; 16 μg/L) [38].

Previous studies focused on the toxicology of several HALs [17]. TCAL was mutagenic in *Salmonella typhimurium* [39-42] and induced chromosomal aberrations [39, 43] and aneuploidy [44, 45] in mammalian cells. TCAL induced micronuclei [46-50], mitotic aberrations [50-53], and DNA strand-breaks [54-56] in mammalian cells. The toxicity of CAL was studied as a metabolite of industrial chemicals such as vinyl chloride [57]. CAL was cytotoxic in rat hepatocytes [58] and induced nephrotoxicity in human renal proximal tubule cells [59]. CAL formed DNA adducts and

caused mutations [60-66]. CAL generated mitotic chromosome malsegregation [67] and interstrand cross-links [68], and DCAL induced mitotic aneuploidy [69]. BAL irreversibly bound to DNA and protein in rat liver microsomes [70]. TBAL induced single- and double-strand DNA breaks [54]. However, systematic toxicological effects of other emerging HAL DBPs were not investigated and there is no quantitative, comparative database on the toxicity of individual HALs.

The objective of our research was to: (i) develop new analytical chemical methods and identify the occurrence of a new HAL DBP in drinking water, (ii) analyze the *in vitro* cytotoxicity and genotoxicity of HALs and related compounds in mammalian cells, (iii) determine the cytotoxicity and genotoxicity index values of HALs and develop a quantitative, comparative toxicity database, and (iv) conduct a mechanism-based structure-activity relationship analysis for the observed HAL mediated cytotoxicity and genotoxicity. In 1974, trihalomethanes (THMs) were discovered as the first chemical class of disinfection by-products (DBPs) in drinking water [2]. Since then, numerous efforts were put onto the DBP research including identification of emerging DBPs, formation kinetics, exposure assessment, risk assessment, toxicology and molecular mechanisms, epidemiological evidence, and evaluation of mixtures. To date, more than 600 DBPs have been identified, and haloacetaldehydes (HALs) are in a class of unregulated emerging DBPs.

3.2. MATERIALS AND METHODS

3.2.1. General Reagents

General reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) and Fisher Scientific Co. (Itasca, IL). Media and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. (Itasca, IL). Chemical standards were purchased from Sigma-Aldrich, ChemService (West Chester, PA), Orchid Cellmark (Westminster, BC, Canada), and TCI America (Waltham, MA) at the highest level of purity.

3.2.2. Preparation of Haloacetaldehydes

Ten HALs were analyzed and their sources and purities are presented in Table 3.1. The chemical structure of each HAL is presented in Figure 3.1. A 2M stock solution of each HAL was prepared in dimethylsulfoxide (DMSO) prior to the toxicological experiments. Stock solutions were immediately stored in sterile glass vials under dark conditions at −20°C.

3.2.3. Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8 was used for the toxicity studies [71-73]. The CHO cells were maintained in Ham's F12 medium containing 5% FBS, 1% antibiotics (100 U/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

3.2.4. CHO Cell Chronic Cytotoxicity Assay

This 96-well microplate assay measures the reduction in cell density as a function of the HAL concentration over a period of 72 h (~3 cell cycles) [15, 32]. Chronic cytotoxicity to CHO cells was measured using an assay we previously developed for the analysis of DBPs [32]. In general, 8 replicates were prepared for each concentration of HAL. Eight wells were reserved for the blank control consisting of 200 µL of F12 medium + 5% FBS. The negative control consisted of 8 wells containing 100 µL of a tittered CHO cell suspension (3×10^4 cells/mL) plus 100 µL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known concentration of HAL for a total volume of 200 µL. To prevent cross-over contamination between wells due to volatilization of the organic extract, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells, and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 10 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed in tap water, inverted and tapped dry upon paper towels, and 50 µL of DMSO/methanol (3:1 v/v) was added to each well for 10 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet on a microcomputer connected to the microplate reader. The blank-corrected absorbance of the negative control (cells with medium only) was set at 100%. The absorbance for each treatment group well was converted into a percentage of the negative control. The experiments were repeated 2-4 times. A concentration-response curve was generated for each HAL and a

regression analysis was conducted for each curve. The LC_{50} values were calculated, where the LC_{50} represents the HAL concentration that induced a 50% reduction in cell density as compared to the concurrent negative controls.

3.2.5. CHO Cell Single Cell Gel Electrophoresis Assay

Single cell gel electrophoresis (SCGE, or Comet) assay quantitatively measures genomic DNA damage in individual nuclei [74-76]. We employed the microplate SCGE method [77]. The SCGE metric for genomic DNA damage induced by the HALs was the %tail DNA value (%TDNA), which is the amount of DNA that migrated from the nucleus into the microgel [78]. The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 µL of F12 + 5% FBS and incubated. The next day, the cells were washed with HBSS and treated with a series of concentrations of HALs in F12 medium without FBS in a total volume of 25 µL for 4 h at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal™. After incubation, the cells were washed 2× with HBSS and harvested with 50 µL of 0.01% trypsin + 53 µM EDTA. The trypsin was inactivated with 70 µL of F12 + FBS. Acute cytotoxicity was measured from a 10 µL aliquot of cell suspension mixed with 10 µL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS) [79]. SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The cellular membranes were removed by an overnight immersion in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) at 4°C. The microgels were

placed in an alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were neutralized with tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis, the microgels were hydrated in cold water for 30 min and stained with 65 µL of ethidium bromide (20 µg/mL) for 3 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, 2 microgels were prepared per treatment group. Randomly chosen nuclei (25 per microgel) were analyzed using a charged coupled device camera. A computerized image analysis system (Comet IV, Perspective Instruments, Ltd, Suffolk, UK) was employed to determine the SCGE %Tail DNA value of the nuclei as indices of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. Within each experiment, a negative control, a positive control (3.8 mM ethylmethanesulfonate), and 9 concentrations of HALs were analyzed concurrently. The experiments were repeated 2-3 times. For each HAL concentration range, a concentration-response curve was generated. A regression analysis was used to fit the curve, and the concentration inducing a 50%Tail DNA value was calculated.

3.2.6. Statistical Analyses

For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to determine if the HAL induced a statistically significant level of cell death at a specific

concentration. If a significant F value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was performed to identify the lowest cytotoxic concentration. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha = 0.05$. For the SCGE assay, the %Tail DNA values are not normally distributed, which limits the use of parametric statistics [80]. The mean %Tail DNA value for each microgel was calculated and these values were averaged among all of the microgels for each HAL concentration. A one-way ANOVA test was conducted on these averaged %Tail DNA values [81]. If a significant *F* value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power ≥ 0.8 at $\alpha = 0.05$. A bootstrap statistical approach was used to generate a series of multiple LC_{50} values and %Tail DNA per each HAL [82, 83]. For each LC*50* value a cytotoxicity index (CTI) value was calculated as $(LC_{50})^{-1}(10^3)$. For each %Tail DNA value a genotoxicity index (GTI) value was calculated as $(50\%\text{Tail DNA})^{-1}(10^3)$. These dimensionless values were then analyzed using an ANOVA test to determine significant differences among the HALs. A Pearson's Product Moment correlation test was conducted to test for correlations among cytotoxicity and genotoxicity data and HAL chemical characteristics.

3.2.7. Haloacetaldehyde Analytical Methods (By Dr. C. Postigo and Dr. S. Richardson)

Two analytical methodologies based on gas chromatography coupled to mass spectrometry were validated to investigate the occurrence of HALs in source and disinfected waters. Mono-halogenated HALs (monoHALs) and di-halogenated HALs (diHALs) were derivatized with pentafluorobenzylhydroxylamine (PFBHA), and subsequently liquid-liquid

extracted. Tri-halogenated HALs (triHALs) was pre-concentrated by means of solid-phase extraction.

3.3. RESULTS

3.3.1. Haloacetaldehyde DBP Analytical Methods and Results (By Dr. C. Postigo and Dr. S. Richardson)

The work on the chemical analysis section was conducted by Dr. Cristina Postigo under the direction of Dr. S. Richardson. The chemical analysis of the source and disinfected water revealed the absence of target HALs in the source waters and the presence of all target HALs in drinking waters. However, trace levels of DBAL, TBAL, DBCAL and IAL were detected in one sample of source waters. The level of IAL in drinking water ranged between 0.6 ppb and 4.6 ppb. Overall, IAL concentrations were similar to those observed for triHALs and DBAL, and slightly higher than those measured for CAL, DCAL and BAL. An example of the identification of IAL is presented in Figure 3.2.

3.3.2. CHO Cell Chronic Cytotoxicity

CHO cell chronic cytotoxicity analyses of each HAL are summarized in Table 3.2. Figure 3.3 illustrates the concentration-response curves for the HALs. The individual concentration-response curves of each HAL are presented in the Figures 3.4 - 3.13). An all pairwise ANOVA test of the CTI values generated a descending rank order of chronic cytotoxicity of the ten HALs

as TBAL ≈ CAL > DBAL ≈ BCAL ≈ DBCAL > IAL > BAL ≈ BDCAL ≈ DCAL > TCAL. The mean bootstrap CTI (±SE) values are presented in Table 3.4 and Figure 3.14.

3.3.3. CHO Cell Acute Genotoxicity

CHO cell acute genotoxicity analyses of each HAL are summarized in Table 3.3. Figure 3.15 illustrates the concentration-response curves for the HALs. The individual concentration-response curves of each HAL with the cell viability data are presented in Figures 3.16-3.25. An all pairwise ANOVA test of the GTI values generated a descending rank order of genotoxicity of the ten HALs as DBAL > CAL ≈ DBCAL > TBAL ≈ BAL ≈ BDCAL ≈ BCAL ≈ DCAL ≈ IAL. TCAL was not genotoxic. The mean bootstrap GTI (±SE) values are presented in Table 3.4 and Figure 3.26.

3.4. DISCUSSION

3.4.1. Structure-Activity Relationships of Haloacetaldehyde Toxicity

The most cytotoxic HALs were TBAL and CAL, followed by DBAL and BCAL. The most genotoxic HAL was DBAL, followed by CAL and DBCAL (Table 3.4 and Figure 3.14). The cytotoxicity and genotoxicity of these 10 HALs were not significantly correlated (r = 0.36; P = 0.308). This response is in contrast to other chemical DBP classes such as haloacetic acids (HAAs) [84], THMs [32], or haloacetamides (HAcAms) [16] where the halogen affected toxicity (iodinated- > brominated- > chlorinated-DBPs).

The toxicity of HALs is complex in that these compounds possess two potential reactive sites to react with nucleophiles in cells. One is the α-carbon (αC)-halogen (X) bond which is

associated with S_N2 type reactions. With the monohalogenated HAAs (monoHAAs) and monoHAcAms, the rank order of toxicity followed I > Br >> Cl, which corresponds to the leaving tendency of the halogens of alkyl halide [16, 84, 85]. We found that the monoHAAs irreversibly inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and that there was a high correlation with the dissociation energy of the αC-X bond and the alkylation potential of the HAA [84, 85]. The rate of inhibition of GAPDH and the toxic potency of the monoHAAs showed a concentration-dependent manner with the same rank order as above. A pattern for the cytotoxicity of the HALs was a rank order for the triHALs was TBAL > DBCAL > BDCAL > TCAL and for the diHALs was DBAL > BCAL > DCAL. With the combined data of triHALs and diHALs, a strong significant correlation was found between the number of Br atoms and the CTI (r = 0.90; $P \leq 0.006$), while a good but not significant correlation was found with the GTI values (r = 0.63; P = 0.13). However, the impact of the halogen was not observed in the monoHALs.

The other reactive site of HALs is the carbonyl C=O bond of the aldehyde group. Aliphatic aldehydes are able to undergo Schiff base formation (Figure 3.27). The Schiff base formation is a mechanism used by enzymes to catalyze reactions between an amine group with either an aldehyde or ketone. It proceeds through the carbinolamine intermediate with an imine as a final product. HALs may induce genotoxic effects such as DNA adducts, DNA-DNA cross-links, or DNA-protein cross-links by reacting with DNA chains through Schiff base formation [60, 65, 66]. Therefore, the overall toxic potency may differ by individual compounds depending on the combinative reactivity of S_N2 type reaction and Schiff base formation in a biological system.

In an aqueous phase, HALs exist in equilibrium between an aldehyde and a hydrate form (Figure 3.28). This hydration equilibrium constant is defined as $K_{hydration}$, with $K_{hydration}$= [hydrate] / [aldehyde]. As $K_{hydration}$ increases, the hydrate species is dominant in the aqueous system. Based on the theoretical $K_{hydration}$ values obtained from a predictive modeling system, SPARC (SPARC Performs Automated Reasoning in Chemistry), which was developed by the U.S. EPA [86, 87], $K_{hydration}$ values increased as the number of halogens increased (Table 3.4). Table 3.4 presents the predicted $K_{hydration}$ values for each HAL analyzed for toxicity. The monoHALs expressed a $K_{hydration}$ that were 2-4 orders of magnitude smaller than the diHALs and triHALs. The halogen-induced toxicity pattern seen with other DBP classes was not expressed in the monoHALs. MonoHALs have distinct $K_{hydration}$ values where the distribution of reactive aldehyde species will differ by halogen type. Therefore, monoHALs may induce overall toxicity outcomes through more than one mode of action. For the di- and triHALs the halogen-mediated S_N2 reaction may perform the predominant role in the induction of toxicity.

3.4.2. Comparison of the Toxicity of Haloacetaldehydes to Other DBP Classes

We compared the cytotoxic and genotoxic potencies among HALs and other DBP chemical classes with their calculated cytotoxicity and genotoxicity indices (Figure 3.29). The cytotoxicity index was determined by calculating the mean $(LC_{50})^{-1}(10^3)$ value of all of the individual compounds of a single class of DBPs. The genotoxicity index was determined by calculating the mean SCGE genotoxic potency value which is defined by the reciprocal of midpoint value of the SCGE tail moment $\times 10^3$ from the individual compounds within a single class of DBPs (SCGE tail moment; the integrated value of DNA density multiplied by the

migration distance) [32]. Total of six DBP chemical classes (THMs, HAAs, HALs, halonitromethanes, haloacetonitriles, and haloacetamides) were compared. The HALs were the second most cytotoxic DBP class, while their genotoxicity ranked the second lowest. However, using SCGE tail moment as the metric for genotoxicity has a possibility to underestimate the genotoxicity by their role as DNA cross-linkers. Formaldehyde and acetaldehyde are well-known DNA cross-linkers. With a similar structure, HALs may form DNA cross-links and such formations will reduce the migration of DNA fragments from the nuclei and result in lower genotoxicity measurements.

3.5. CONCLUSION

The HALs are the third largest group by weight of identified DBPs formed in drinking water. This study identified a new HAL DBP, IAL, and showed that HALs are cytotoxic and genotoxic to mammalian cells. This study provided the first systematic, quantitative comparison of HAL toxicity. The most cytotoxic HALs were TBAL and CAL, followed by DBAL and BCAL. The most genotoxic HAL was DBAL, followed by CAL and DBCAL. HALs were highly cytotoxic among other DBP chemical classes. Therefore, HALs may adversely affect the public health and the environment and further research are needed to investigate the mode of action of its toxicity.

References

1. Cutler, D.; Miller, G., The role of public health improvements in health advances: the twentieth-century United States. *Demography* **2005,** *42*, (1), 1-22.
2. Rook, J. J., Formation of haloforms during chlorination of natural waters *J. Soc. Water Treat. Exam.* **1974,** *23*, 234-243.
3. Bond, T.; Huang, J.; Templeton, M. R.; Graham, N., Occurrence and control of nitrogenous disinfection by-products in drinking water- a review. *Water Res.* **2011,** *45*, (15), 4341-4354.
4. Chu, W.; Gao, N.; Yin, D.; Krasner, S. W., Formation and speciation of nine haloacetamides, an emerging class of nitrogenous DBPs, during chlorination or chloramination. *J. Hazard. Mater.* **2013,** *260*, 806-812.
5. Krasner, S. W.; Mitch, W. A.; McCurry, D. L.; Hanigan, D.; Westerhoff, P., Formation, precursors, control, and occurrence of nitrosamines in drinking water: a review. *Water Res.* **2013,** *47*, (13), 4433-4450.
6. Weinberg, H. S., Modern approaches to the analysis of disinfection by-products in drinking water. *Philos. Trans. A Math. Phys. Eng. Sci.* **2009,** *367*, (1904), 4097-4118.
7. Kimura, S. Y.; Komaki, Y.; Plewa, M. J.; Marinas, B. J., Chloroacetonitrile and N,2-dichloroacetamide formation from the reaction of chloroacetaldehyde and monochloramine in water. *Environ. Sci. Technol.* **2013,** *47*, (21), 12382-12390.
8. Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. *Philos. Transact. A Math. Phys. Eng. Sci.* **2009,** *367*, (1904), 4077-4095.
9. Pereira, R. O.; Postigo, C.; de Alda, M. L.; Daniel, L. A.; Barcelo, D., Removal of estrogens through water disinfection processes and formation of by-products. *Chemosphere* **2011,** *82*, (6), 789-799.
10. Feder, P. I.; Ma, Z. J.; Bull, R. J.; Teuschler, L. K.; Schenck, K. M.; Simmons, J. E.; Rice, G., Evaluating sufficient similarity for disinfection by-product (DBP) mixtures: multivariate statistical procedures. *J. Toxicol. Environ. Health A* **2009,** *72*, (7), 468-481.
11. Grellier, J.; Bennett, J.; Patelarou, E.; Smith, R. B.; Toledano, M. B.; Rushton, L.; Briggs, D. J.; Nieuwenhuijsen, M. J., Exposure to disinfection by-products, fetal growth, and prematurity: a systematic review and meta-analysis. *Epidemiology* **2010,** *21*, (3), 300-313.
12. Hrudey, S. E., Chlorination disinfection by-products, public health risk tradeoffs and me. *Water. Res.* **2009,** *43*, (8), 2057-2092.
13. Legay, C.; Rodriguez, M. J.; Serodes, J. B.; Levallois, P., Estimation of chlorination by-products presence in drinking water in epidemiological studies on adverse reproductive outcomes: a review. *Sci. Total. Environ.* **2010,** *408*, (3), 456-472.
14. Ngwenya, N.; Ncube, E. J.; Parsons, J., Recent advances in drinking water disinfection: successes and challenges. *Rev. Environ. Contam. Toxicol.* **2013,** *222*, 111-170.
15. Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2002,** *40*, (2), 134-142.

16. Plewa, M. J.; Muellner, M. G.; Richardson, S. D.; Fasano, F.; Buettner, K. M.; Woo, Y. T.; McKague, A. B.; Wagner, E. D., Occurrence, synthesis, and mammalian cell cytotoxicity and genotoxicity of haloacetamides: an emerging class of nitrogenous drinking water disinfection byproducts. *Environ. Sci. Technol.* **2008,** *42*, (3), 955-961.
17. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636*, (1-3), 178-242.
18. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286*, (1-3), 1-19.
19. Costet, N.; Villanueva, C. M.; Jaakkola, J. J.; Kogevinas, M.; Cantor, K. P.; King, W. D.; Lynch, C. F.; Nieuwenhuijsen, M. J.; Cordier, S., Water disinfection by-products and bladder cancer: is there a European specificity? A pooled and meta-analysis of European case-control studies. *Occup. Environ. Med.* **2011,** *68*, (5), 379-385.
20. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39*, (3), 733-745.
21. Villanueva, C. M.; Cantor, K. P.; Cordier, S.; Jaakkola, J. J.; King, W. D.; Lynch, C. F.; Porru, S.; Kogevinas, M., Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* **2004,** *15*, (3), 357-367.
22. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.
23. Costet, N.; Garlantezec, R.; Monfort, C.; Rouget, F.; Gagniere, B.; Chevrier, C.; Cordier, S., Environmental and urinary markers of prenatal exposure to drinking water disinfection by-products, fetal growth, and duration of gestation in the PELAGIE birth cohort (Brittany, France, 2002-2006). *Am. J. Epidemiol.* **2012,** *175*, (4), 263-275.
24. Grazuleviciene, R.; Nieuwenhuijsen, M. J.; Vencloviene, J.; Kostopoulou-Karadanelli, M.; Krasner, S. W.; Danileviciute, A.; Balcius, G.; Kapustinskiene, V., Individual exposures to drinking water trihalomethanes, low birth weight and small for gestational age risk: a prospective Kaunas cohort study. *Environ. Health* **2011,** *10*, 32.
25. Hinckley, A. F.; Bachand, A. M.; Reif, J. S., Late pregnancy exposures to disinfection by-products and growth-related birth outcomes. *Environ. Health Perspect.* **2005,** *113*, (12), 1808-1813.
26. Jeong, C. H.; Wagner, E. D.; Siebert, V. R.; Anduri, S.; Richardson, S. D.; Daiber, E. J.; McKague, A. B.; Kogevinas, M.; Villanueva, C. M.; Goslan, E. H.; Luo, W.; Isabelle, L. M.; Pankow, J. F.; Grazuleviciene, R.; Cordier, S.; Edwards, S. C.; Righi, E.; Nieuwenhuijsen, M. J.; Plewa, M. J., The occurrence and toxicity of disinfection byproducts in European drinking waters in relation with the HIWATE epidemiology study. *Envion. Sci. Technol.* **2012,** *46*, (21), 12120-12128.

27. Villanueva, C. M.; Gracia-Lavedan, E.; Ibarluzea, J.; Santa Marina, L.; Ballester, F.; Llop, S.; Tardon, A.; Fernandez, M. F.; Freire, C.; Goni, F.; Basagana, X.; Kogevinas, M.; Grimalt, J. O.; Sunyer, J.; Project, I., Exposure to trihalomethanes through different water uses and birth weight, small for gestational age, and preterm delivery in Spain. *Environ. Health Perspect.* **2011,** *119*, (12), 1824-1830.
28. Wright, J. M.; Schwartz, J.; Dockery, D. W., Effect of trihalomethane exposure on fetal development. *Occup. Environ. Med.* **2003,** *60*, (3), 173-180.
29. Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ. Res.* **2007,** *104*, (3), 390-395.
30. Hunter, E. S.; Rogers, E. H.; Schmid, J. E.; Richard, A., Comparative effects of haloacetic acids in whole embryo culture. *Teratology* **1996,** *54*, (2), 57-64.
31. Narotsky, M. G.; Best, D. S.; McDonald, A.; Godin, E. A.; Hunter, E. S., 3rd; Simmons, J. E., Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. *Reprod. Toxicol.* **2011,** *31*, (1), 59-65.
32. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
33. Wei, X.; Wang, S.; Zheng, W.; Wang, X.; Liu, X.; Jiang, S.; Pi, J.; Zheng, Y.; He, G.; Qu, W., Drinking water disinfection byproduct iodoacetic acid induces tumorigenic transformation of NIH3T3 cells. *Environ. Sci. Technol.* **2013,** *47*, (11), 5913-5920.
34. Richardson, S. D., Postigo, C., The handbook of Environmental Chemistry: Emerging Organic Contaminants and Human Health. In *Drinking Water Disinfection By-Products*, Springer: New York, NY, 2012; pp 93-137.
35. Xie, Y., *Disinfection Byproducts in Drinking Water; Formation, Analysis, and Control*. CRC Press/Lewis Publishers: Boca Raton, FL, 2004.
36. McGuire, M. J.; McLain, J. L.; Obolensky, A., *Information Collection Rute Data Analysis*. AwwaRF and AWWA: Denver, CO, 2002.
37. Koudjonou, B.; Lebel, G. L.; Dabeka, L., Formation of halogenated acetaldehydes, and occurrence in Canadian drinking water. *Chemosphere* **2008,** *72*, (6), 875-881.
38. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., The occurrence of a new generation of disinfection by-products. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-7185.
39. Beland, F. A., NTP technical report on the toxicity and metabolism studies of chloral hydrate (CAS No. 302-17-0). Administered by gavage to F344/N rats and B6C3F1 mice. *Toxic. Rep. Ser.* **1999**, (59), 1-66, A1-E7.
40. Haworth, S.; Lawlor, T.; Mortelmans, K.; Speck, W.; Zeiger, E., Salmonella mutagenicity test results for 250 chemicals. *Environ. Mutagen.* **1983,** *5 Suppl 1*, 1-142.
41. Leuschner, J.; Leuschner, F., Evaluation of the mutagenicity of chloral hydrate in vitro and in vivo. *Arzneim.-Forsch.* **1991,** *41*, (10), 1101-1103.
42. Ni, Y. C.; Wong, T. Y.; Kadlubar, F. F.; Fu, P. P., Hepatic metabolism of chloral hydrate to free radical(s) and induction of lipid peroxidation. *Biochem. Biophys. Res. Commun.* **1994,** *204*, (2), 937-943.

43. Furnus, C. C.; Ulrich, M. A.; Terreros, M. C.; Dulout, F. N., The induction of aneuploidy in cultured Chinese hamster cells by propionaldehyde and chloral hydrate. *Mutagenesis* **1990,** *5*, (4), 323-326.
44. Mailhes, J. B.; Preston, R. J.; Yuan, Z. P.; Payne, H. S., Analysis of mouse metaphase II oocytes as an assay for chemically induced aneuploidy. *Mutat. Res.* **1988,** *198*, (1), 145-152.
45. Russo, A.; Pacchierotti, F.; Metalli, P., Nondisjunction induced in mouse spermatogenesis by chloral hydrate, a metabolite of trichloroethylene. *Environ. Mutagen.* **1984,** *6*, (5), 695-703.
46. Allen, J. W.; Collins, B. W.; Evansky, P. A., Spermatid micronucleus analyses of trichloroethylene and chloral hydrate effects in mice. *Mutat. Res.* **1994,** *323*, (1-2), 81-88.
47. Migliore, L.; Nieri, M., Evaluation of twelve potential aneuploidogenic chemicals by the in vitro human lymphocyte micronucleus assay. *Toxicol. In Vitro* **1991,** *5*, (4), 325-336.
48. Nesslany, F.; Marzin, D., A micromethod for the in vitro micronucleus assay. *Mutagenesis* **1999,** *14*, (4), 403-410.
49. Parry, J. M.; Parry, E. M.; Bourner, R.; Doherty, A.; Ellard, S.; O'Donovan, J.; Hoebee, B.; de Stoppelaar, J. M.; Mohn, G. R.; Onfelt, A.; Renglin, A.; Schultz, N.; Soderpalm-Berndes, C.; Jensen, K. G.; Kirsch-Volders, M.; Elhajouji, A.; Van Hummelen, P.; Degrassi, F.; Antoccia, A.; Cimini, D.; Izzo, M.; Tanzarella, C.; Adler, I. D.; Kliesch, U.; Hess, P.; et al., The detection and evaluation of aneugenic chemicals. *Mutat. Res.* **1996,** *353*, (1-2), 11-46.
50. Parry, J. M.; Parry, E. M.; Warr, T.; Lynch, A.; James, S., The detection of aneugens using yeasts and cultured mammalian cells. *Prog. Clin. Biol. Res.* **1990,** *340B*, 247-266.
51. Brunner, M.; Albertini, S.; Wurgler, F. E., Effects of 10 known or suspected spindle poisons in the in vitro porcine brain tubulin assembly assay. *Mutagenesis* **1991,** *6*, (1), 65-70.
52. Eichenlaub-Ritter, U.; Baart, E.; Yin, H.; Betzendahl, I., Mechanisms of spontaneous and chemically-induced aneuploidy in mammalian oogenesis: basis of sex-specific differences in response to aneugens and the necessity for further tests. *Mutat. Res.* **1996,** *372*, (2), 279-294.
53. Warr, T. J.; Parry, E. M.; Parry, J. M., A comparison of two in vitro mammalian cell cytogenetic assays for the detection of mitotic aneuploidy using 10 known or suspected aneugens. *Mutat. Res.* **1993,** *287*, (1), 29-46.
54. Liviac, D.; Creus, A.; Marcos, R., DNA damage induction by two halogenated acetaldehydes, byproducts of water disinfection. *Water Res.* **2010,** *44*, (8), 2638-2646.
55. Moore, M. M.; Harrington-Brock, K., Mutagenicity of trichloroethylene and its metabolites: implications for the risk assessment of trichloroethylene. *Environ. Health Perspect.* **2000,** *108 Suppl 2*, 215-223.
56. Nelson, M. A.; Bull, R. J., Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver in vivo. *Toxicol. Appl. Pharmacol.* **1988,** *94*, (1), 45-54.
57. Guengerich, F. P.; Crawford, W. M., Jr.; Watanabe, P. G., Activation of vinyl chloride to covalently bound metabolites: roles of 2-chloroethylene oxide and 2-chloroacetaldehyde. *Biochemistry* **1979,** *18*, (23), 5177-5182.

58. Sood, C.; O'Brien, P. J., Molecular mechanisms of chloroacetaldehyde-induced cytotoxicity in isolated rat hepatocytes. *Biochem. Pharmacol.* **1993,** *46*, (9), 1621-1626.
59. Benesic, A.; Schwerdt, G.; Mildenberger, S.; Freudinger, R.; Gordjani, N.; Gekle, M., Disturbed Ca2+-signaling by chloroacetaldehyde: A possible cause for chronic ifosfamide nephrotoxicity. *Kidney. Int.* **2005,** *68*, (5), 2029-2041.
60. Biernat, J.; Ciesiolka, J.; Gornicki, P.; Adamiak, R. W.; Kryzosiak, W. J.; Wiewiorowski, M., New observations concerning the chloroacetaldehyde reaction with some tRNA constituents. Stable intermediates, kinetics and selectivity of the reaction. *Nucleic Acids Res.* **1978,** *5*, (3), 789-804.
61. Chiang, S. Y.; Swenberg, J. A.; Weisman, W. H.; Skopek, T. R., Mutagenicity of vinyl chloride and its reactive metabolites, chloroethylene oxide and chloroacetaldehyde, in a metabolically competent human B-lymphoblastoid line. *Carcinogenesis* **1997,** *18*, (1), 31-36.
62. Dosanjh, M. K.; Chenna, A.; Kim, E.; Fraenkel-Conrat, H.; Samson, L.; Singer, B., All four known cyclic adducts formed in DNA by the vinyl chloride metabolite chloroacetaldehyde are released by a human DNA glycosylase. *Proc. Natl. Acad. Sci. USA* **1994,** *91*, (3), 1024-1028.
63. Hansen, R. J.; Nagasubramanian, R.; Delaney, S. M.; Samson, L. D.; Dolan, M. E., Role of O6-methylguanine-DNA methyltransferase in protecting from alkylating agent-induced toxicity and mutations in mice. *Carcinogenesis* **2007,** *28*, (5), 1111-1116.
64. Jacobsen, J. S.; Humayun, M. Z., Mechanisms of mutagenesis by the vinyl chloride metabolite chloroacetaldehyde. Effect of gene-targeted in vitro adduction of M13 DNA on DNA template activity in vivo and in vitro. *Biochemistry* **1990,** *29*, (2), 496-504.
65. Palejwala, V. A.; Simha, D.; Humayun, M. Z., Mechanisms of mutagenesis by exocyclic DNA adducts. Transfection of M13 viral DNA bearing a site-specific adduct shows that ethenocytosine is a highly efficient RecA-independent mutagenic noninstructional lesion. *Biochemistry* **1991,** *30*, (36), 8736-8743.
66. Pandya, G. A.; Moriya, M., 1,N6-ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells. *Biochemistry* **1996,** *35*, (35), 11487-11492.
67. Crebelli, R.; Conti, G.; Conti, L.; Carere, A., Chloroacetaldehyde Is a Powerful Inducer of Mitotic Aneuploidy in Aspergillus-Nidulans. *Mutagenesis* **1990,** *5*, (2), 165-168.
68. Spengler, S. J.; Singer, B., Formation of interstrand cross-links in chloroacetaldehyde-treated DNA demonstrated by ethidium bromide fluorescence. *Cancer Research* **1988,** *48*, (17), 4804-4806.
69. Crebelli, R.; Conti, G.; Conti, L.; Carere, A., Induction of Somatic Segregation by Halogenated Aliphatic-Hydrocarbons in Aspergillus-Nidulans. *Mutat. Res.* **1984,** *138*, (1), 33-38.
70. Guengerich, F. P.; Mason, P. S.; Stott, W. T.; Fox, T. R.; Watanabe, P. G., Roles of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res.* **1981,** *41*, (11 Pt 1), 4391-4398.

71. Hsie, A. W.; Brimer, P. A.; Mitchell, T. J.; Gosslee, D. G., The dose-response relationship for ultraviolet-light-induced mutations at the hypoxanthine-guanine phosphoribosyltransferase locus in Chinese hamster ovary cells. *Somatic Cell Genet.* **1975,** *1*, (4), 383-389.
72. Tindall, K. R.; Stankowski, L. F., Jr.; Machanoff, R.; Hsie, A. W., Detection of deletion mutations in pSV2gpt-transformed cells. *Mol. Cell Biol.* **1984,** *4*, (7), 1411-1415.
73. Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J., Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998,** *32*, (4), 360-368.
74. Fairbairn, D. W.; Olive, P. L.; O'Neill, K. L., The comet assay: a comprehensive review. *Mutat. Res.* **1995,** *339*, (1), 37-59.
75. Rundell, M. S.; Wagner, E. D.; Plewa, M. J., The comet assay: genotoxic damage or nuclear fragmentation? *Environ. Mol. Mutagen.* **2003,** *42*, (2), 61-67.
76. Tice, R. R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J. C.; Sasaki, Y. F., Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **2000,** *35*, (3), 206-221.
77. Wagner, E. D.; Plewa, M. J., Microplate-based comet assay. In *The Comet Assay in Toxicology*, Dhawan, A.; Anderson, D., Eds. Royal Society of Chemistry: London, 2009; pp 79-97.
78. Kumaravel, T. S.; Jha, A. N., Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat. Res.* **2006,** *605*, (1-2), 7-16.
79. Phillips, H. J., Dye exclusion tests for cell viability. In *Tissue Culture: Methods and Applications*, Kruse, P. F.; Patterson, M. J., Eds. Academic Press: New York, 1973; p 406.
80. Box, G. E. P.; Hunter, W. G.; Hunter, J. S., *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*. Wiley & Sons Inc.: New York, NY., 1978.
81. Lovell, D. P.; Omori, T., Statistical issues in the use of the comet assay. *Mutagenesis* **2008,** *23*, (3), 171-182.
82. Efron, B., Better bootstrap confidence intervals *J. Am. Statis. Assoc.* **1987,** *82*, (397), 171-185.
83. Singh, K. X., M. , Bootstrap: A Statistical Method In Rutgers University, 2008; p 18.
84. Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2010,** *51*, (8-9), 871-878.
85. Pals, J. A.; Ang, J. K.; Wagner, E. D.; Plewa, M. J., Biological mechanism for the toxicity of haloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2011,** *45*, (13), 5791-5797.
86. S.H., H.; L.L., B.; L.A., C., Hydration equilibrium constants of aldehydes, ketones, and quinazolines. *QSAR Comb. Sci.* **2005,** *24*, 631-638.
87. U.S.EPA SPARC Performs Automated Reasoning in Chemistry. http://www.epa.gov/extrmurl/research/sparc.html

TABLES AND FIGURES

Table 3.1. Name, sources and purities of haloacetaldehydes analyzed in this study.

Haloactaldehyde	Abbreviation	CASN	Molecular Weight	Source	Purity (%)
Chloroacetaldehyde	CAL	107-20-0	78.50	CanSyn Chem. Co	>95
Bromoacetaldehyde	BAL	17157-48-1	122.95	AldLab Chem.	95
Iodoacetaldehyde	IAL	57782-51-62	169.95	AldLab Chem.	95
Dichloroacetaldehyde	DCAL	79-02-7	112.94	CanSyn Chem. Co	>95
Dibromoacetaldehyde	DBAL	3039-13-2	201.85	CanSyn Chem. Co	>95
Bromochloroacetaldehyde	BCAL	98136-99-3	157.39	CanSyn Chem. Co	85
Trichloroacetaldehyde	TCAL	75-87-06	147.39	CanSyn Chem. Co	>95
Tribromoacetaldehyde	TBAL	115-17-3	280.74	CanSyn Chem. Co	97
Bromodichloroacetaldehyde	BDCAL	34619-29-9	191.84	CanSyn Chem. Co	93
Dibromochloroacetaldehyde	DBCAL	64316-11-6	236.29	CanSyn Chem. Co	87

Table 3.2. Summary of the CHO cell chronic cytotoxicity of the haloacetaldehydes.

Name (Abbrev.)	Conc. Range (μM)	Lowest Cytotoxic Conc.(μM) [a]	LC_{50} (μM) [b]	r^2 [c]	ANOVA Test Statistic [d]
Chloroacetaldehyde (CAL)	0 – 7	0.5	3.51	0.99	$F_{11, 176} = 241$; $P \leq 0.001$
Dichloroacetaldehyde (DCAL)	0 – 15	8	29.25	0.91	$F_{20, 335} = 37.5$; $P \leq 0.001$
Trichloroacetaldehyde (TCAL)	0 – 1600	375	1163	0.94	$F_{24, 333} = 34.0$; $P \leq 0.001$
Bromoacetaldehyde (BAL)	0 – 42	8	17.28	0.98	$F_{23, 248} = 76.1$; $P \leq 0.001$
Dibromoacetaldehyde (DBAL)	0 – 6	2	4.7	0.99	$F_{10, 177} = 165$; $P \leq 0.001$
Tribromoacetaldehyde (TBAL)	0 – 10	2	3.58	0.99	$F_{15, 316} = 256$; $P \leq 0.001$
Iodoacetaldehyde (IAL)	0 – 10	5	6.00	0.96	$F_{12, 163} = 79.6$; $P \leq 0.001$
Bromochloroacetaldehyde (BCAL)	0 – 10	2.5	5.34	0.97	$F_{14, 169} = 31.5$; $P \leq 0.001$
Bromodichloroacetaldehyde (BDCAL)	0 – 50	10	20.35	0.89	$F_{14, 209} = 23.4$; $P \leq 0.001$
Dibromochloroacetaldehyde (DBCAL)	0 – 10	4	5.15	0.95	$F_{16, 167} = 36.1$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest concentration of the haloacetaldehyde in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls. [b] The LC_{50} value is the concentration of the haloacetaldehyde, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. [c] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 3.3. Summary of the CHO cell acute genotoxicity of the haloacetaldehydes.

Name (Abbrev.)	Conc. Range (μM)	Lowest %TDNA Genotoxic Conc. [a] (μM)	50% TDNA [b] (μM)	r^2 [c]	ANOVA Test Statistic [d]
Chloroacetaldehyde (CAL)	0 – 500	100	142.8	0.99	$F_{10, 59} = 62.6$; $P \leq 0.001$
Dichloroacetaldehyde (DCAL)	0 – 2000	800	795	0.98	$F_{19, 60} = 64.0$; $P \leq 0.001$
Trichloroacetaldehyde (TCAL)	0 – 5000	NS	NS	NS	$F_{20, 37} = 0.556$; $P = 0.918$
Bromoacetaldehyde (BAL)	0 – 550	200	381.2	0.98	$F_{10, 68} = 57.2$; $P \leq 0.001$
Dibromoacetaldehyde (DBAL)	0 – 300	50	111.3	0.98	$F_{9, 44} = 41.5$; $P \leq 0.001$
Tribromoacetaldehyde (TBAL)	0 – 500	100	340.3	0.99	$F_{11, 64} = 168$; $P \leq 0.001$
Iodoacetaldehyde (IAL)	0 – 1000	900	1009	0.98	$F_{13, 103} = 22.5$; $P \leq 0.001$
Bromochloroacetaldehyde (BCAL)	0 – 700	500	621.4	0.92	$F_{10, 51} = 22.0$; $P \leq 0.001$
Bromodichloroacetaldehyde (BDCAL)	0 – 600	300	470.4	0.91	$F_{17, 106} = 16.4$; $P \leq 0.001$
Dibromochloroacetaldehyde (DBCAL)	0 – 220	100	143.7	0.99	$F_{5, 29} = 34.4$; $P \leq 0.001$

[a] The lowest genotoxic concentration was the lowest concentration of the haloacetaldehyde in the concentration-response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [b] The SCGE 50% Tail DNA value is the haloacetaldehyde concentration determined from a regression analyses of the data that was calculated to induce a 50% SCGE Tail DNA value. [c] r^2 is the coefficient of determination for the regression analysis upon which the SCGE % Tail DNA value was calculated. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 3.4. Comparison of calculated $K_{hydration}$ values, cytotoxicity index (CTI) values, and genotoxicity index (GTI) values of haloacetaldehydes.

Name (Abbrev.)	SPARC [a] $K_{hydration}$	CTI (±SE) [b]	GTI (±SE) [c]
Chloroacetaldehyde (CAL)	17.8	279.0±7.0	7.20±0.42
Bromoacetaldehyde (BAL)	11.0	64.6±3.5	2.68±0.11
Iodoacetaldehyde (IAL)	4.37	170.4±7.3	0.96±0.03
Dichloroacetaldehyde (DCAL)	1.95×10^3	35.7±0.8	1.26±0.03
Dibromoacetaldehyde (DBAL)	1.58×10^3	207.5±2.1	9.11±0.60
Bromochloroacetaldehyde (BCAL)	1.70×10^3	207.4±11.0	1.61±0.21
Trichloroacetaldehyde (TCAL)	3.24×10^4	0.94±0.03	NS
Tribromoacetaldehyde (TBAL)	1.15×10^4	279.8±4.8	3.00±0.03
Bromodichloroacetaldehyde (BDCAL)	4.37×10^4	51.1±4.3	2.24±0.05
Dibromochloroacetaldehyde (DBCAL)	2.00×10^4	200.2±1.4	6.99±0.28

[a] SPARC (SPARC Performs Automated Reasoning in Chemistry) models are mechanistic perturbation models developed by the U.S. EPA to calculate chemical reactivity and physical processes for compounds from molecular structure [85, 86]. [b] The Cytotoxicity index (CTI) value was calculated from the individual LC_{50} values generated from the bootstrap analyses. The mean CTI was calculated as the $(LC_{50})^{-1}(10^3)$. [c] The Genotoxicity index (GTI) value was calculated from the individual 50%TDNA values generated from the bootstrap analyses. The mean GTI was calculated as the $(LC_{50})^{-1}(10^3)$. A Pearson correlation analysis demonstrated that no significant correlation exists among the hydration constants and the CTI or the GTI.

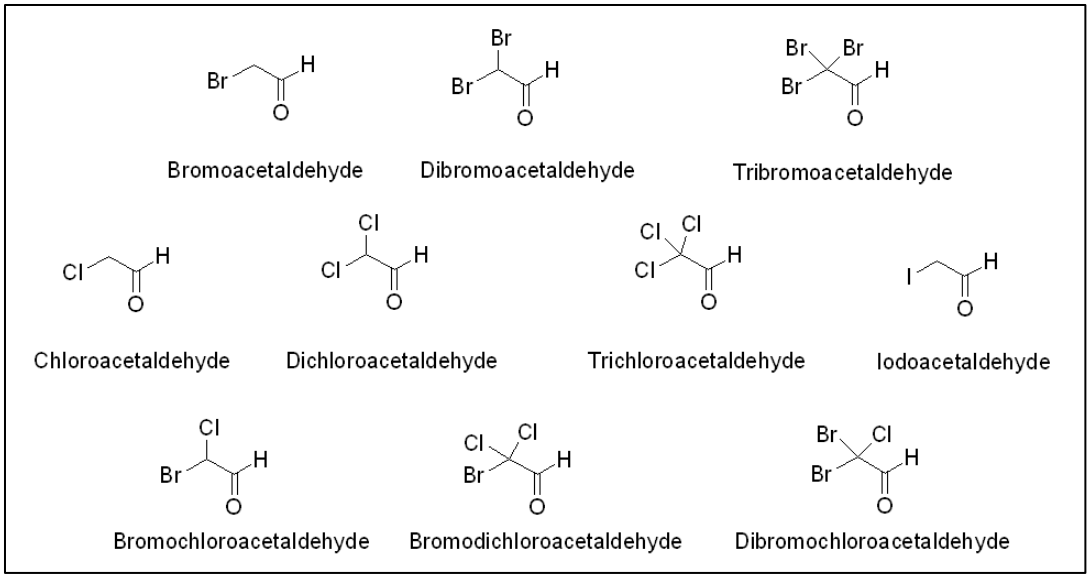


Figure 3.1. Chemical structures of the haloacetaldehydes analyzed in this study.

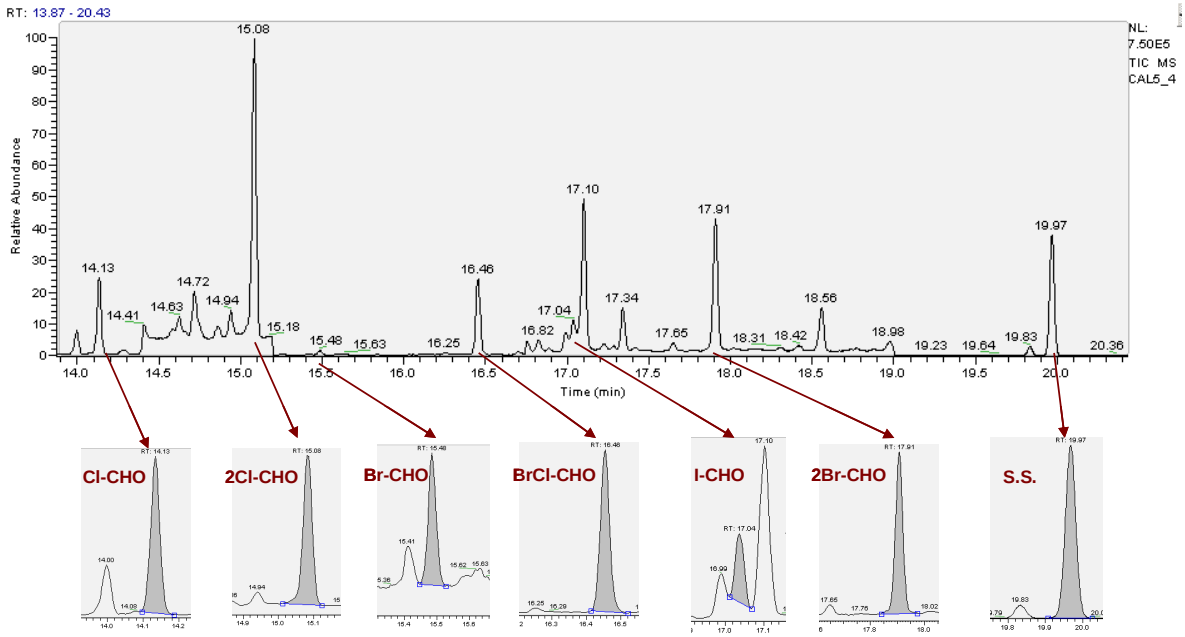


Figure 3.2. . Analyte signal after derivatization-LLE extraction-GC-MS analysis of MiliQ water spiked at a level of 0.5 ppb at a level of 1 ppb for all compounds but for IAL (spiked at 4 ppb).

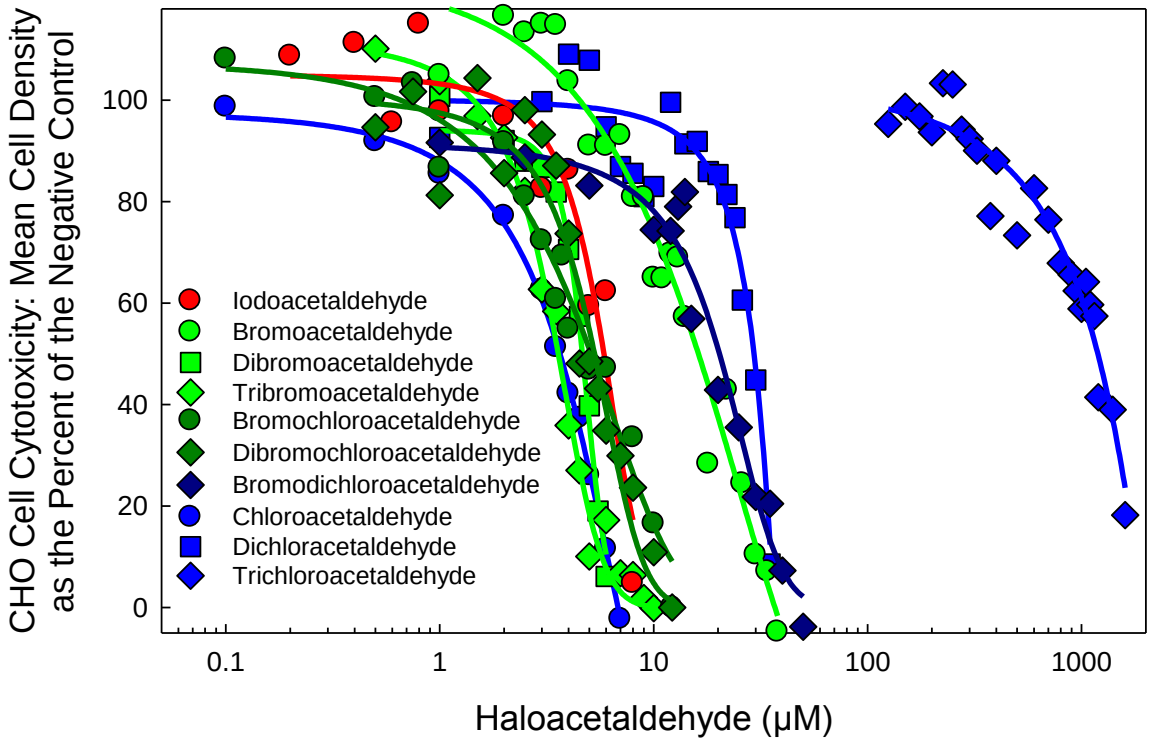


Figure 3.3. Comparison of the CHO cell chronic cytotoxicity concentration-response curves of ten haloacetaldehydes.

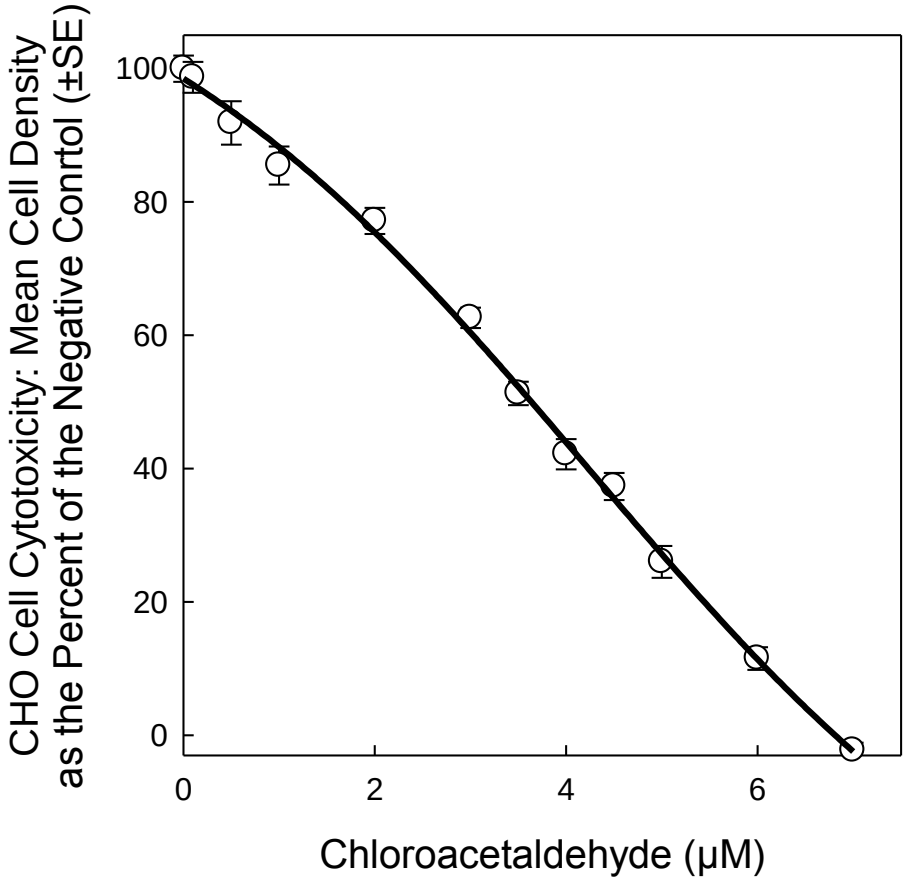


Figure 3.4. Chloroacetaldehyde CHO cell chronic cytotoxicity.

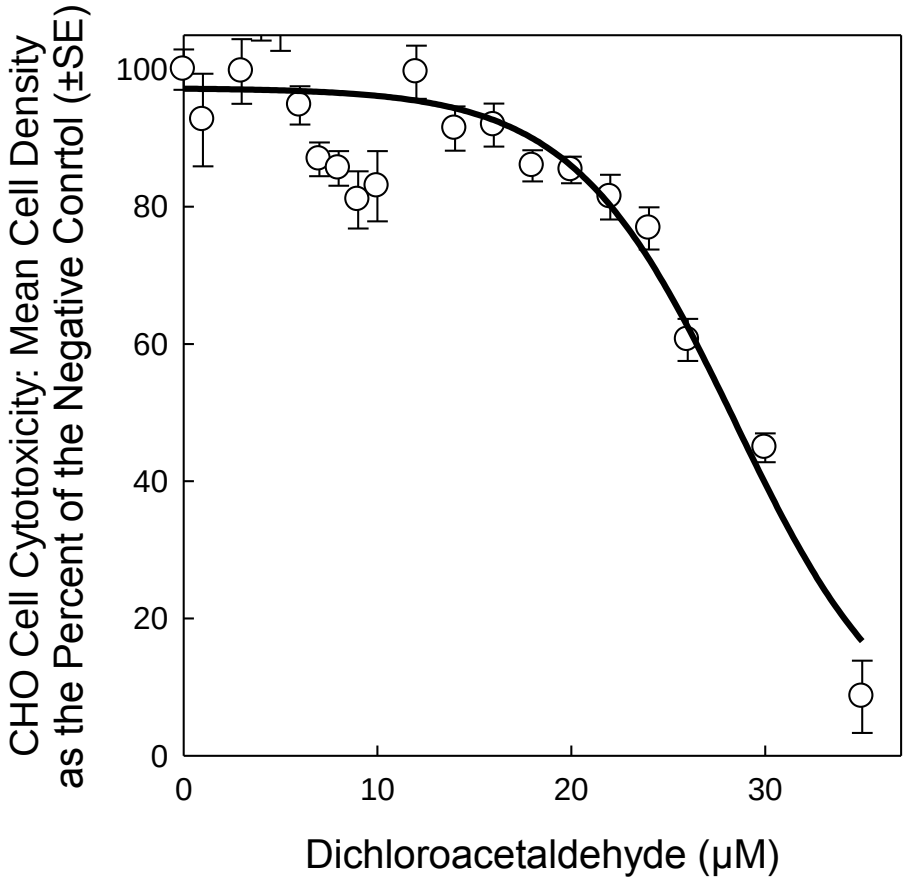


Figure 3.5. Dichloroacetaldehyde CHO cell chronic cytotoxicity.

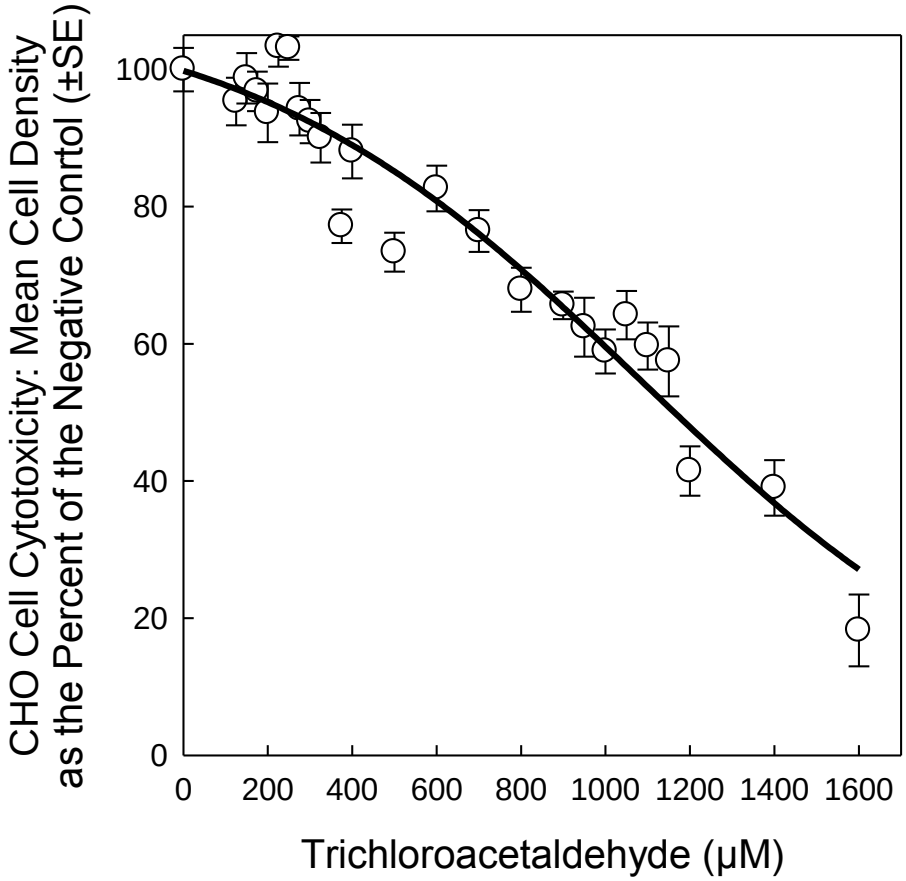


Figure 3.6. Trichloroacetaldehyde CHO cell chronic cytotoxicity.

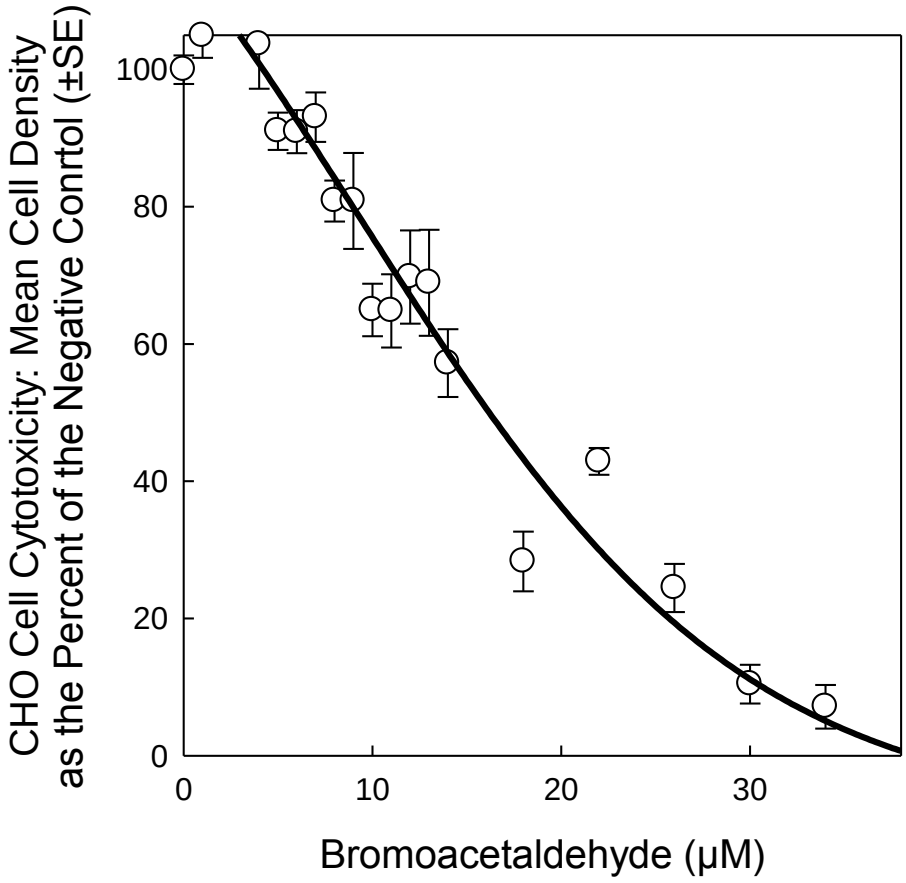


Figure 3.7. Bromoacetaldehyde CHO cell chronic cytotoxicity.

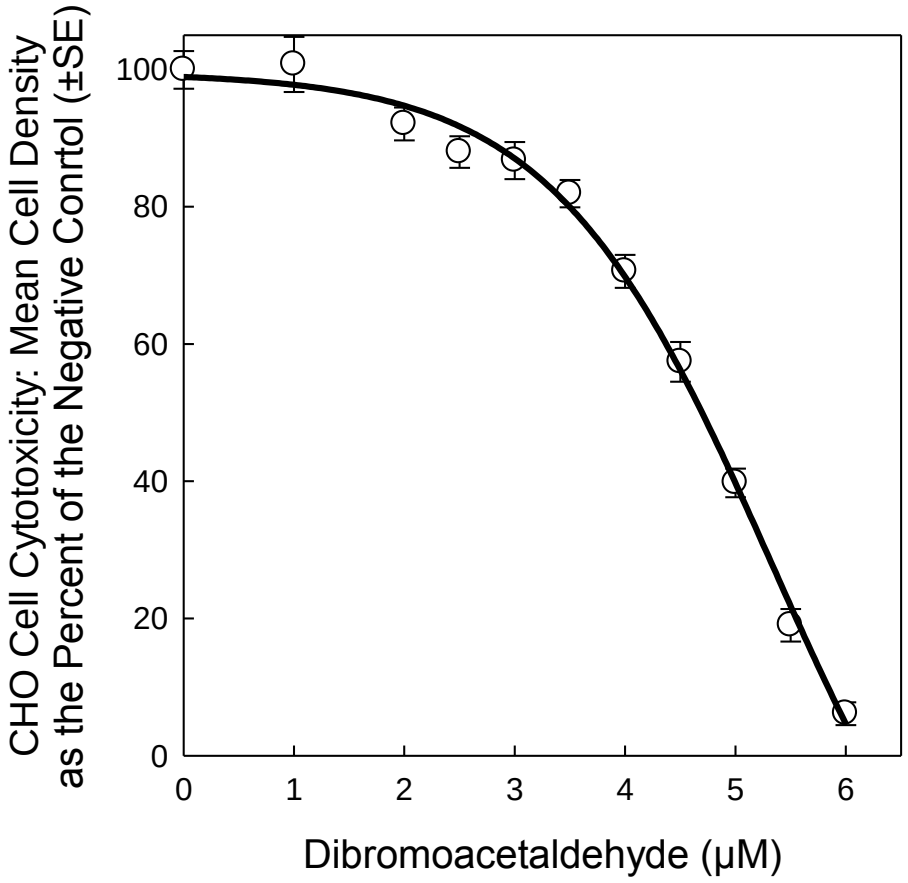


Figure 3.8. Dibromoacetaldehyde CHO cell chronic cytotoxicity.

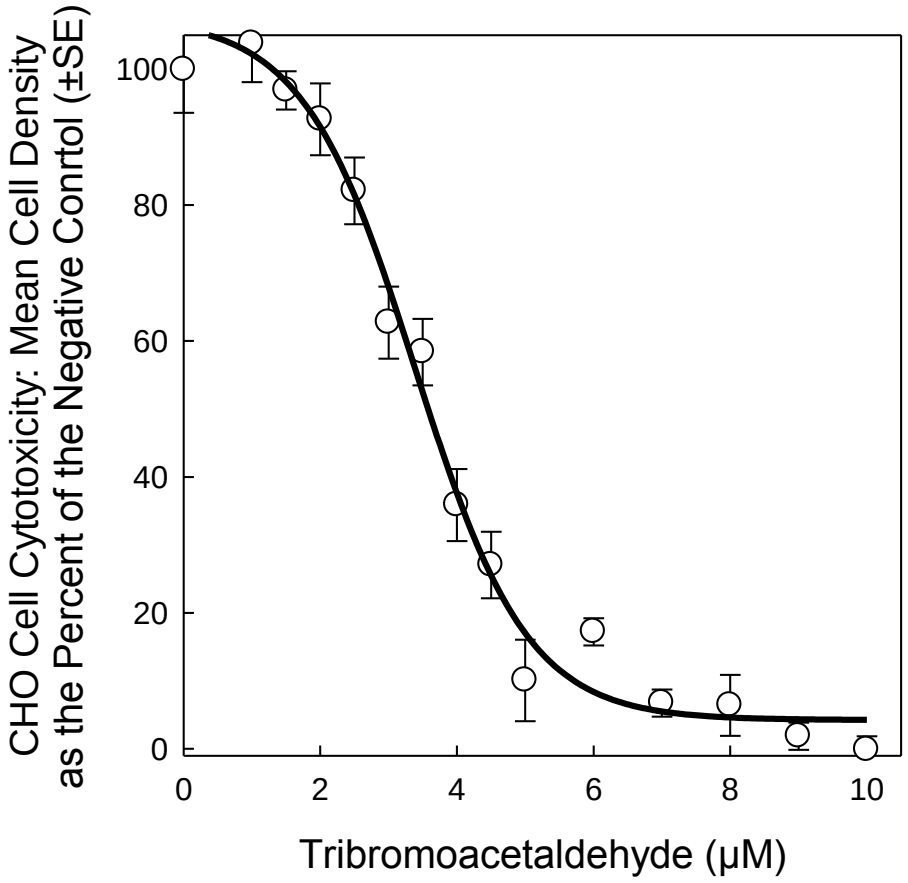


Figure 3.9. Tribromoacetaldehyde CHO cell chronic cytotoxicity.

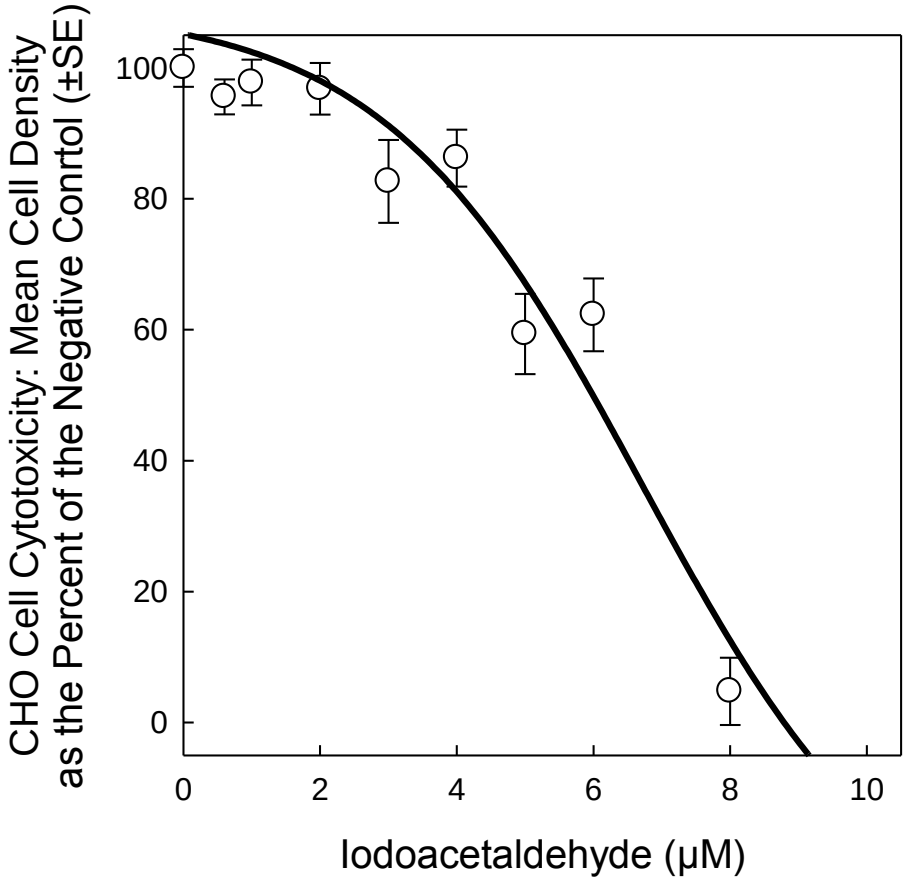


Figure 3.10. Iodoacetaldehyde CHO cell chronic cytotoxicity.

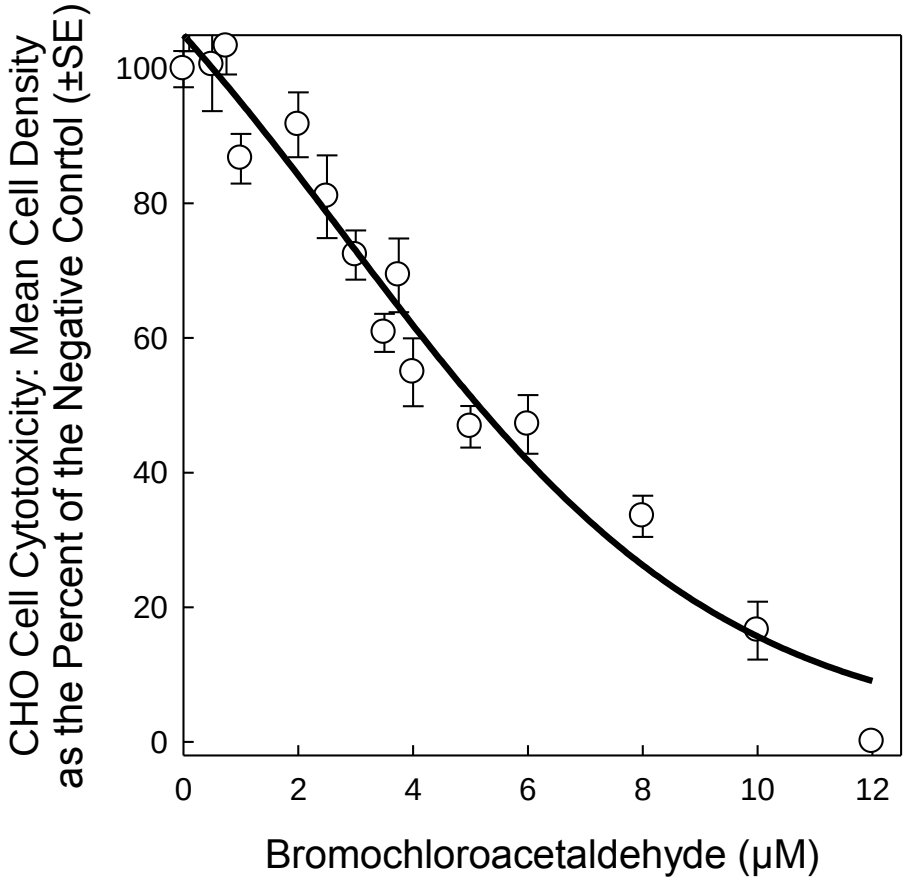


Figure 3.11. Bromochloroacetaldehyde CHO cell chronic cytotoxicity.

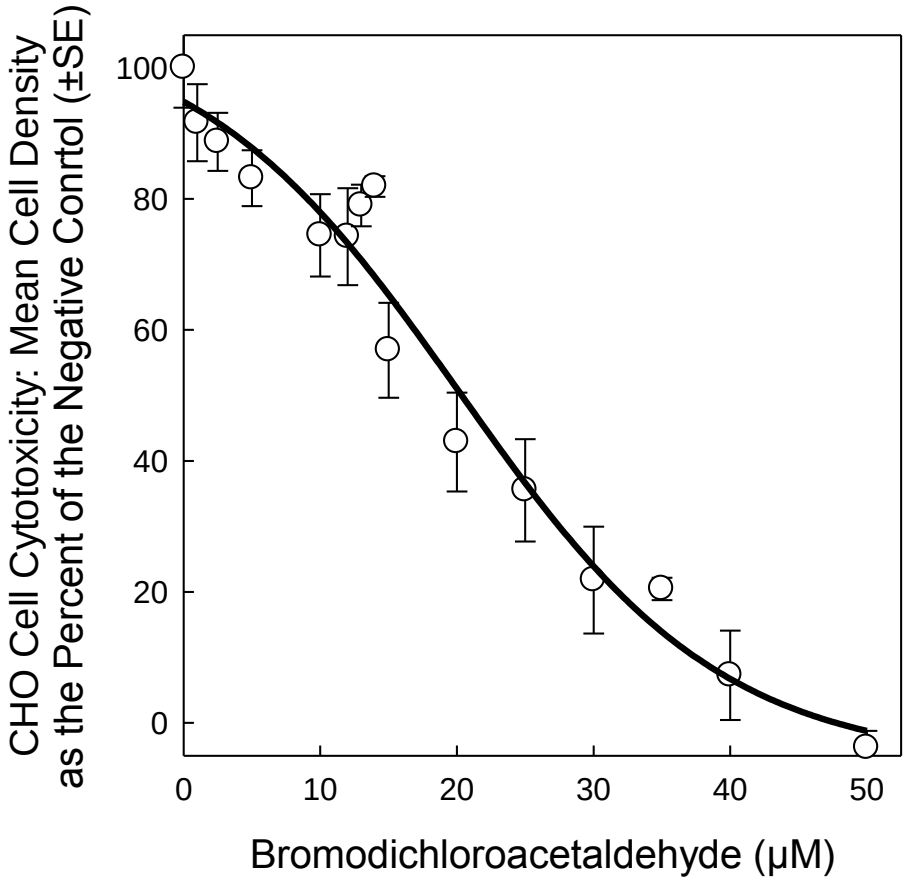


Figure 3.12. Bromodichloroacetaldehyde CHO cell chronic cytotoxicity.

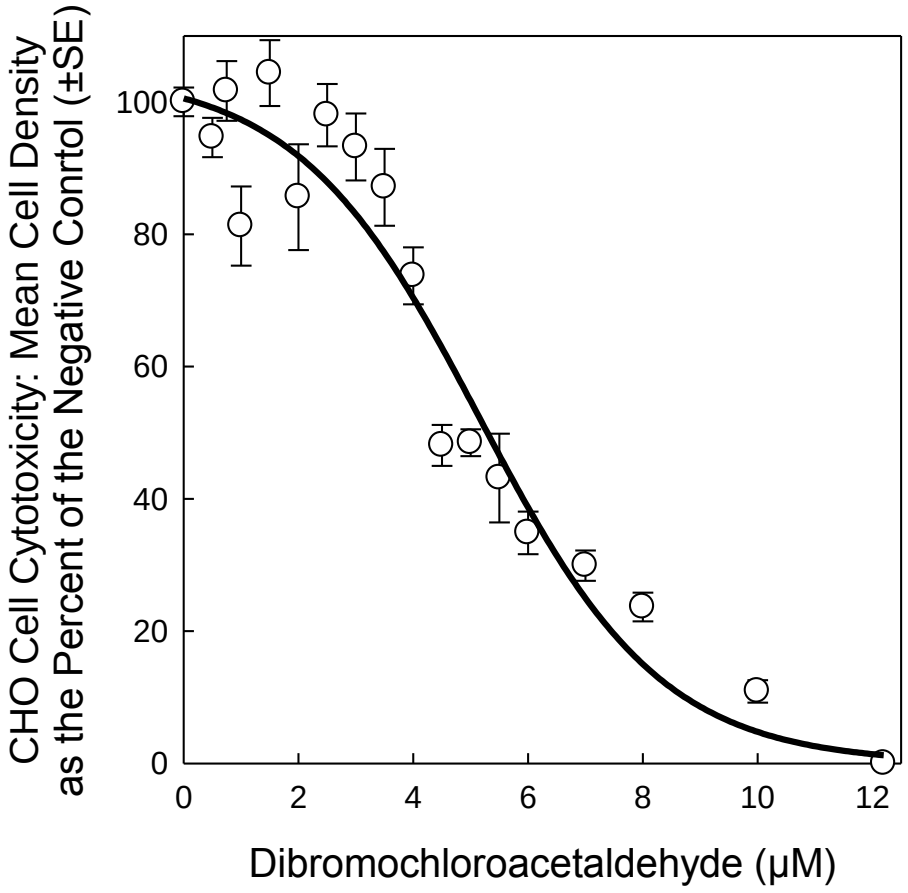


Figure 3.13. Dibromochloroacetaldehyde CHO cell chronic cytotoxicity.

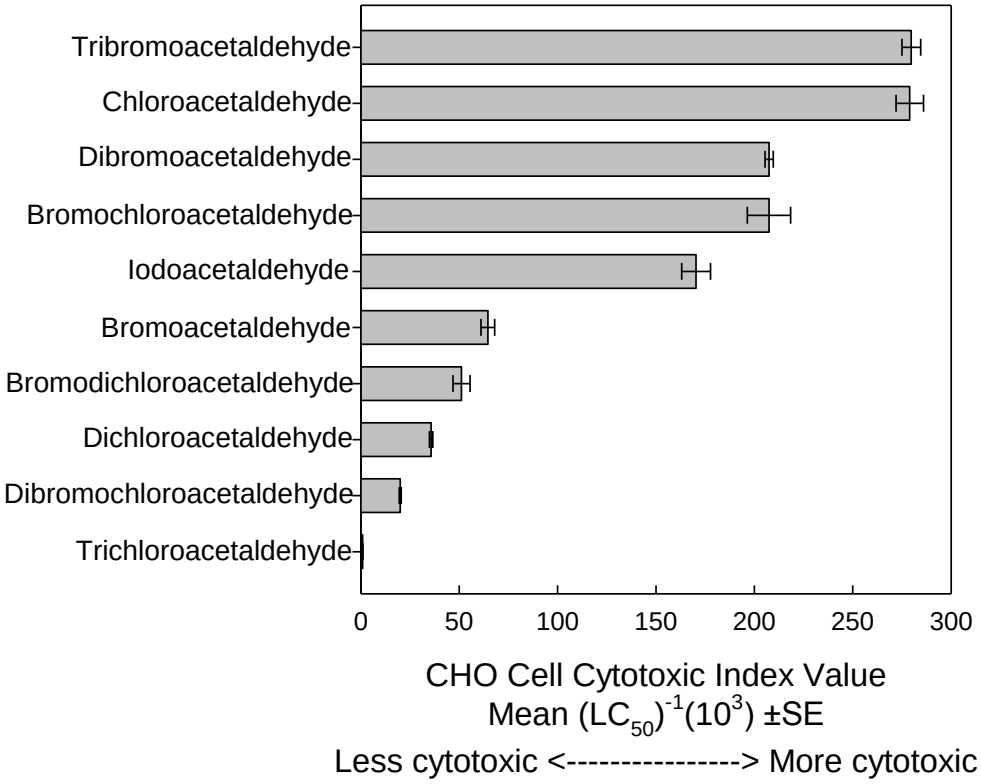


Figure 3.14. The mean bootstrap CHO Cell Cytotoxic Index (CTI) values of ten haloacetaldehydes.

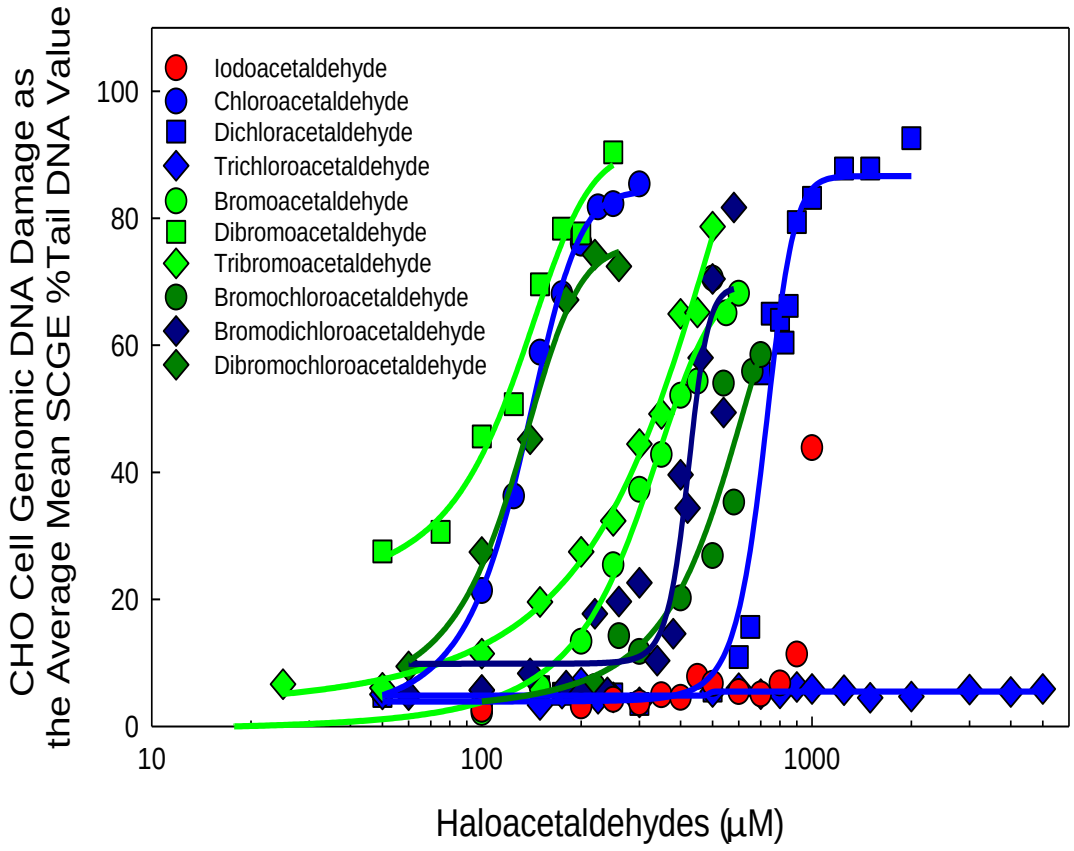


Figure 3.15. Comparison of the CHO cell acute genotoxicity concentration-response curves of ten haloacetaldehydes.

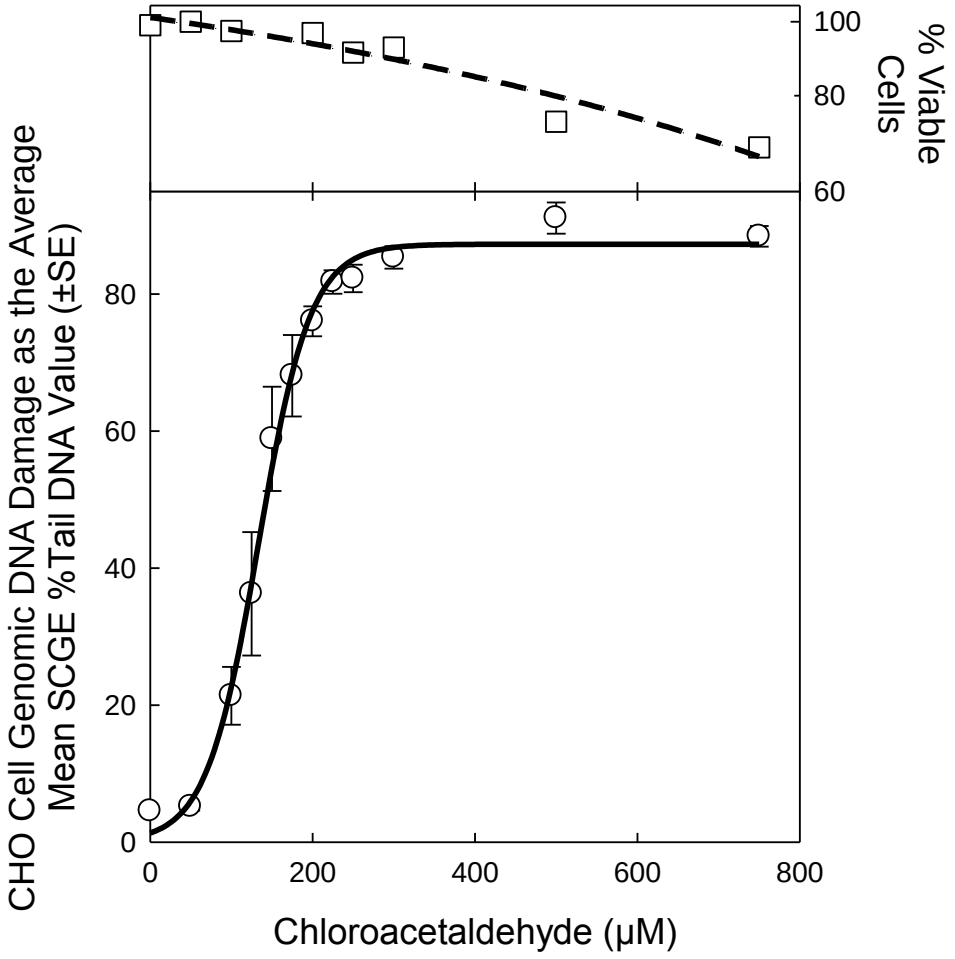


Figure 3.16. Chloroacetaldehyde CHO cell genotoxicity.

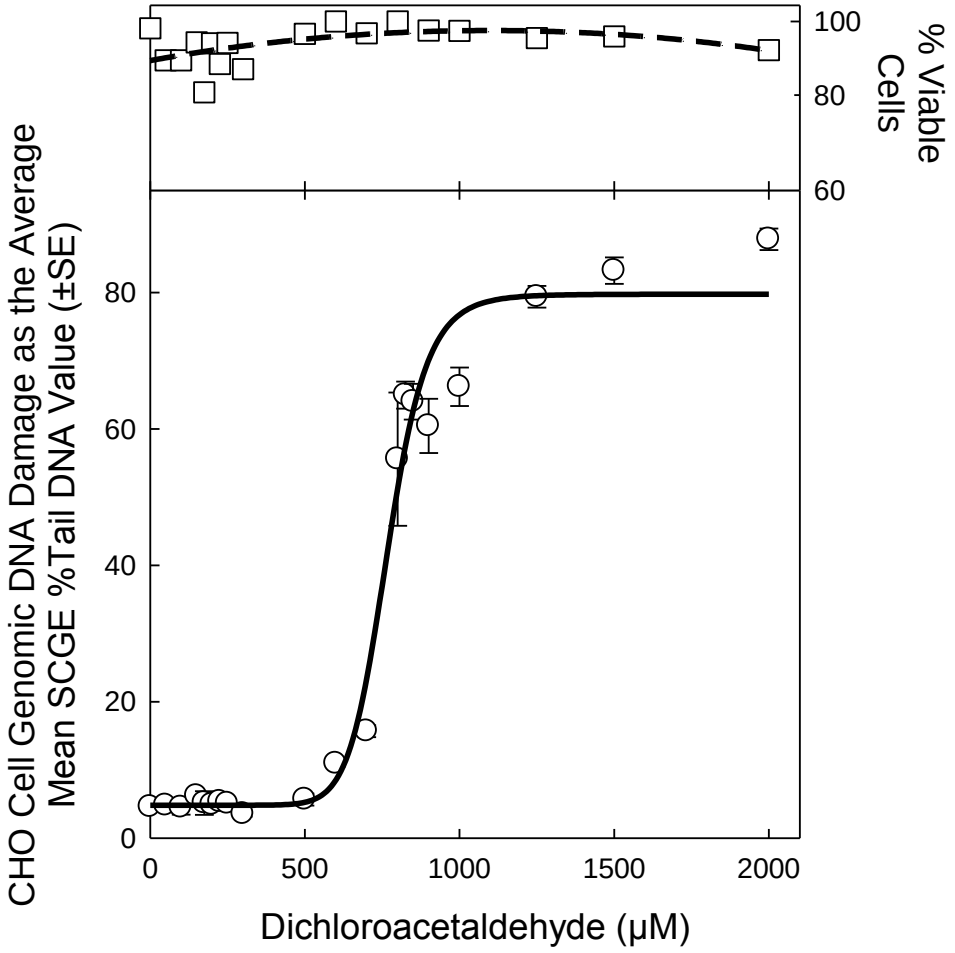


Figure 3.17. Dichloroacetaldehyde CHO cell genotoxicity.

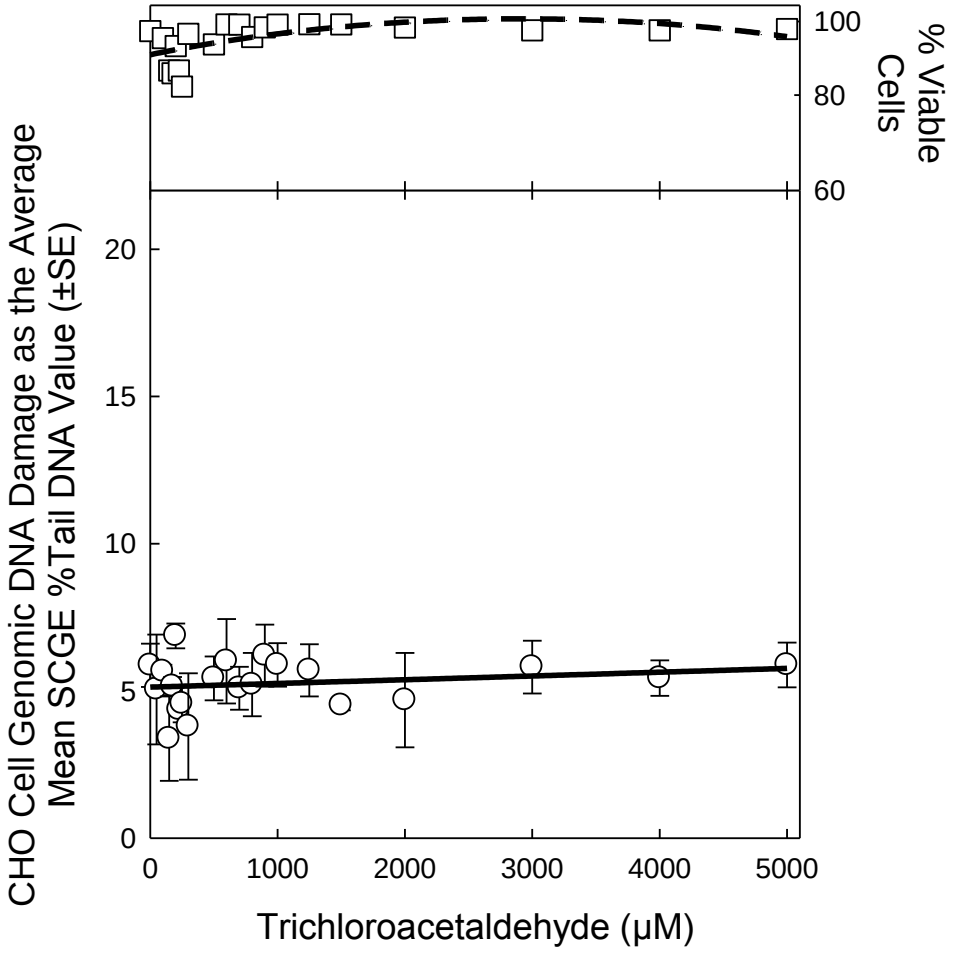


Figure 3.18. Trichloroacetaldehyde CHO cell genotoxicity.

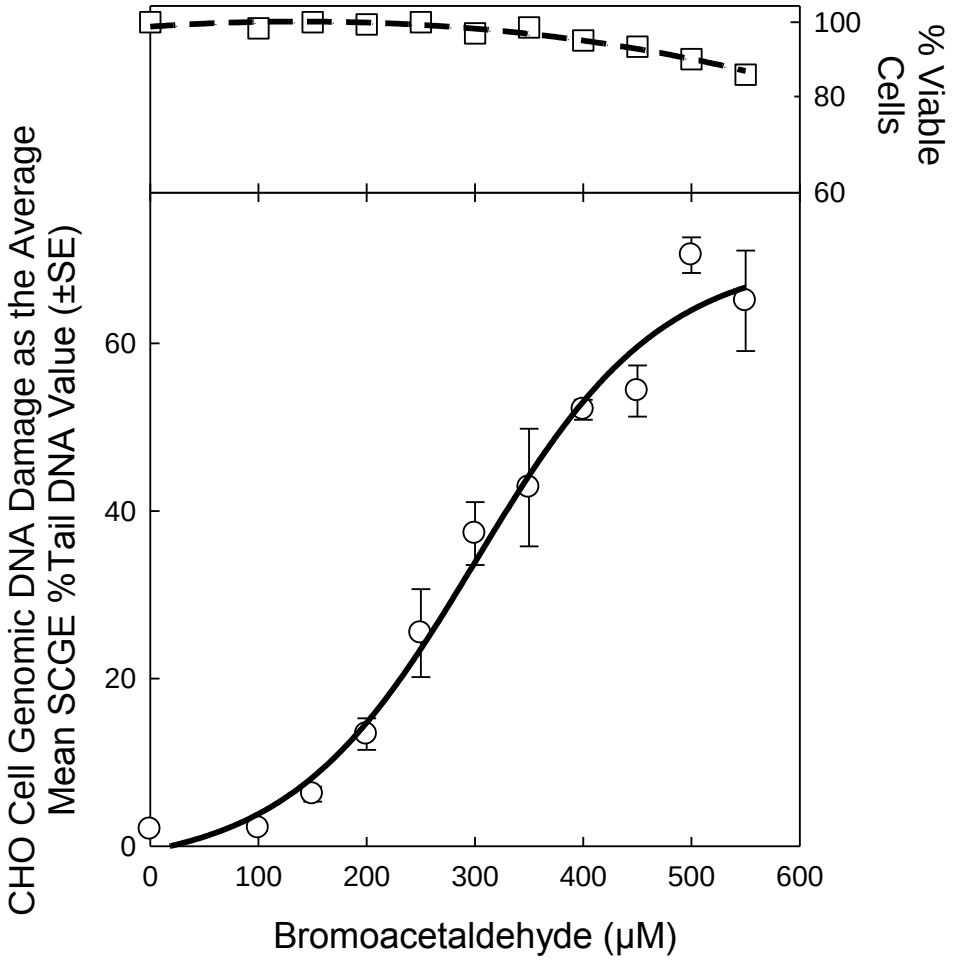


Figure 3.19. Bromoacetaldehyde CHO cell genotoxicity.

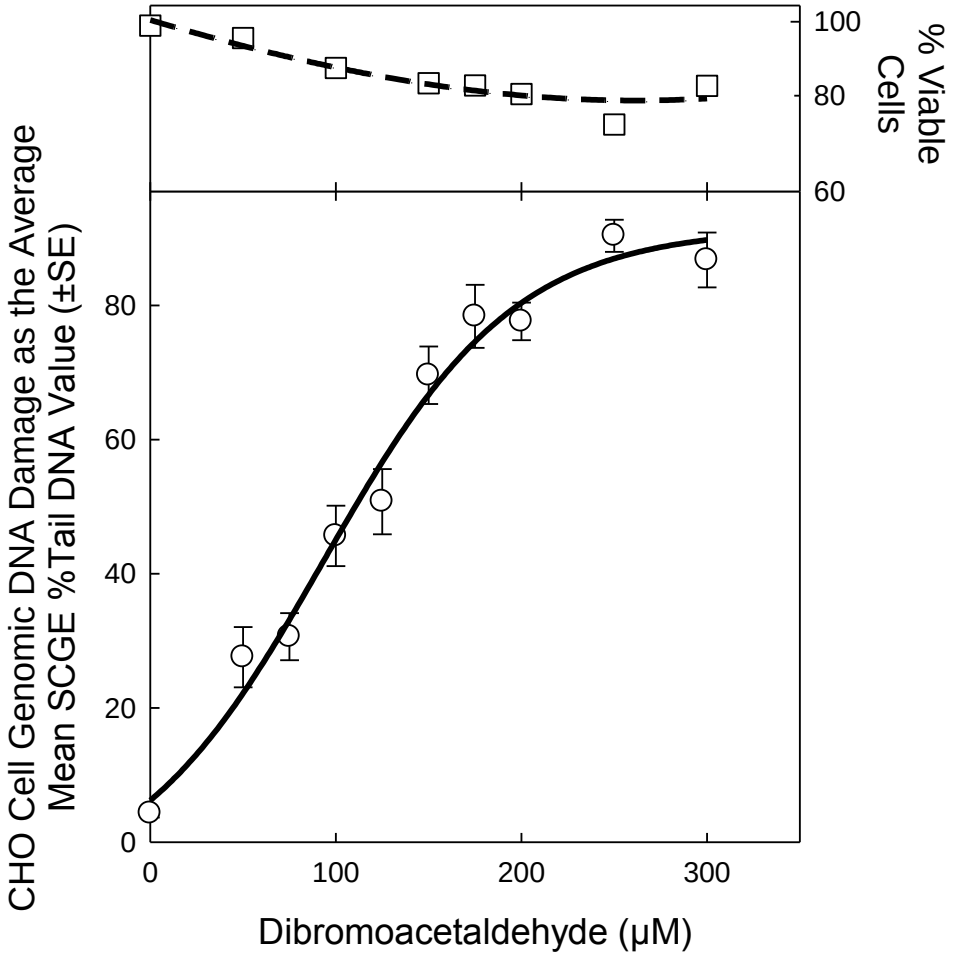


Figure 3.20. Dibromoacetaldehyde CHO cell genotoxicity.

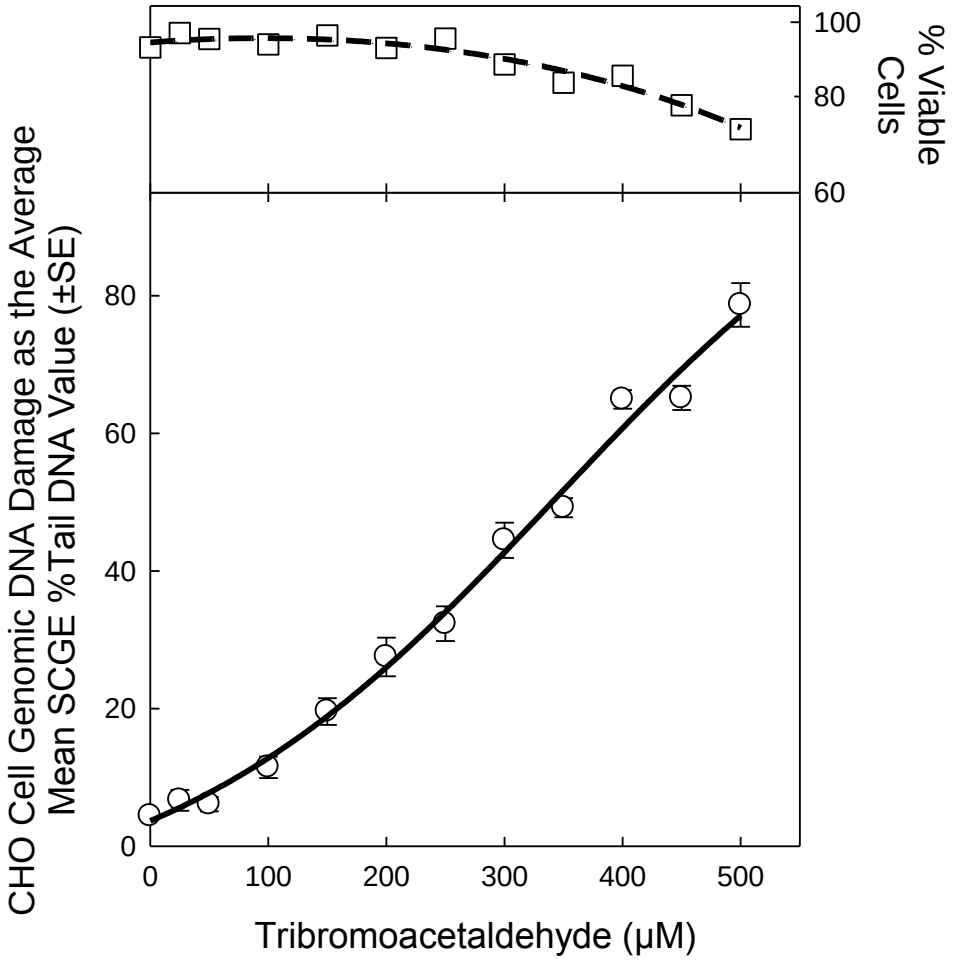


Figure 3.21. Tribromoacetaldehyde CHO cell genotoxicity.

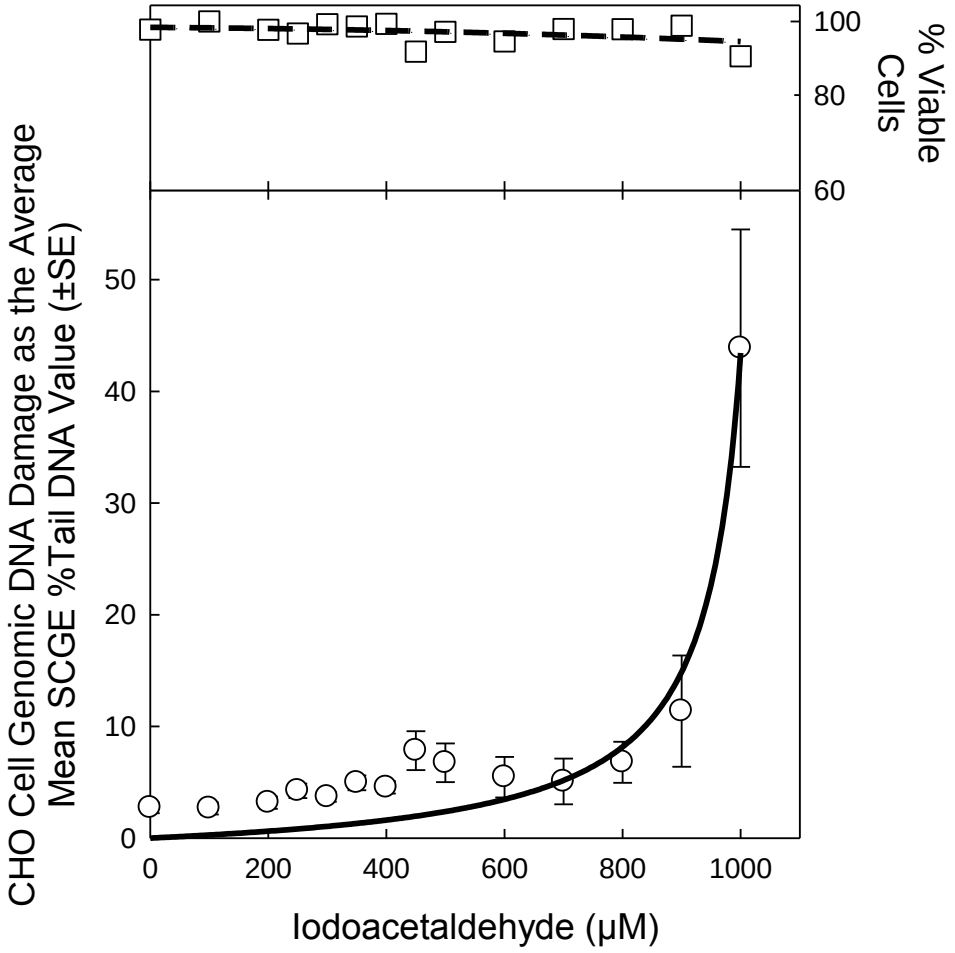


Figure 3.22. Iodoacetaldehyde CHO cell genotoxicity.

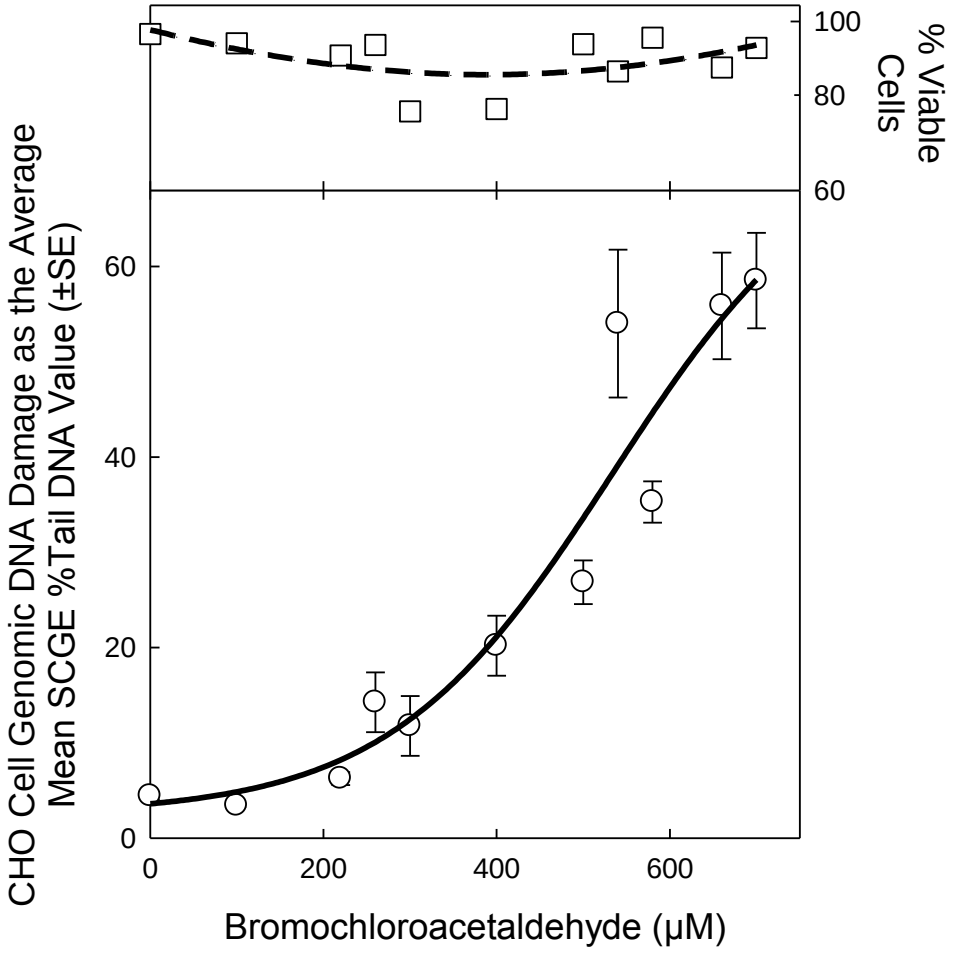


Figure 3.23. Bromochloroacetaldehyde CHO cell genotoxicity.

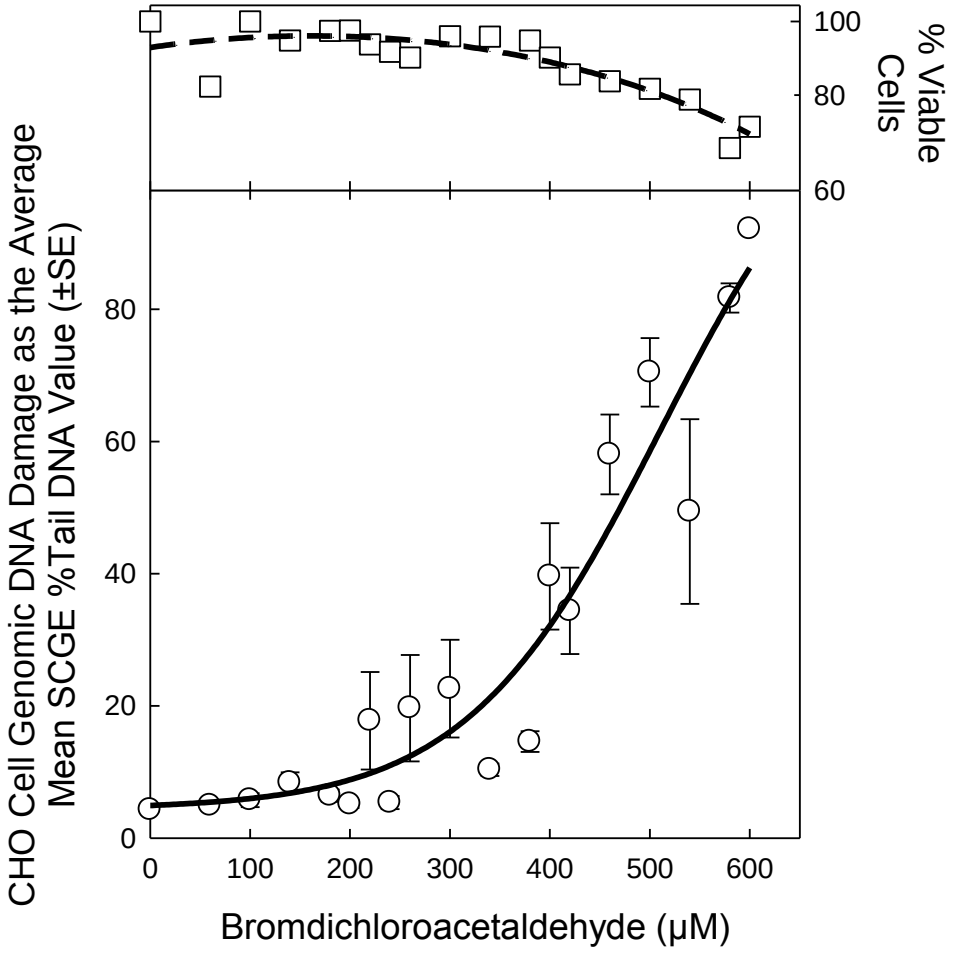


Figure 3.24. Bromodichloroacetaldehyde CHO cell genotoxicity.

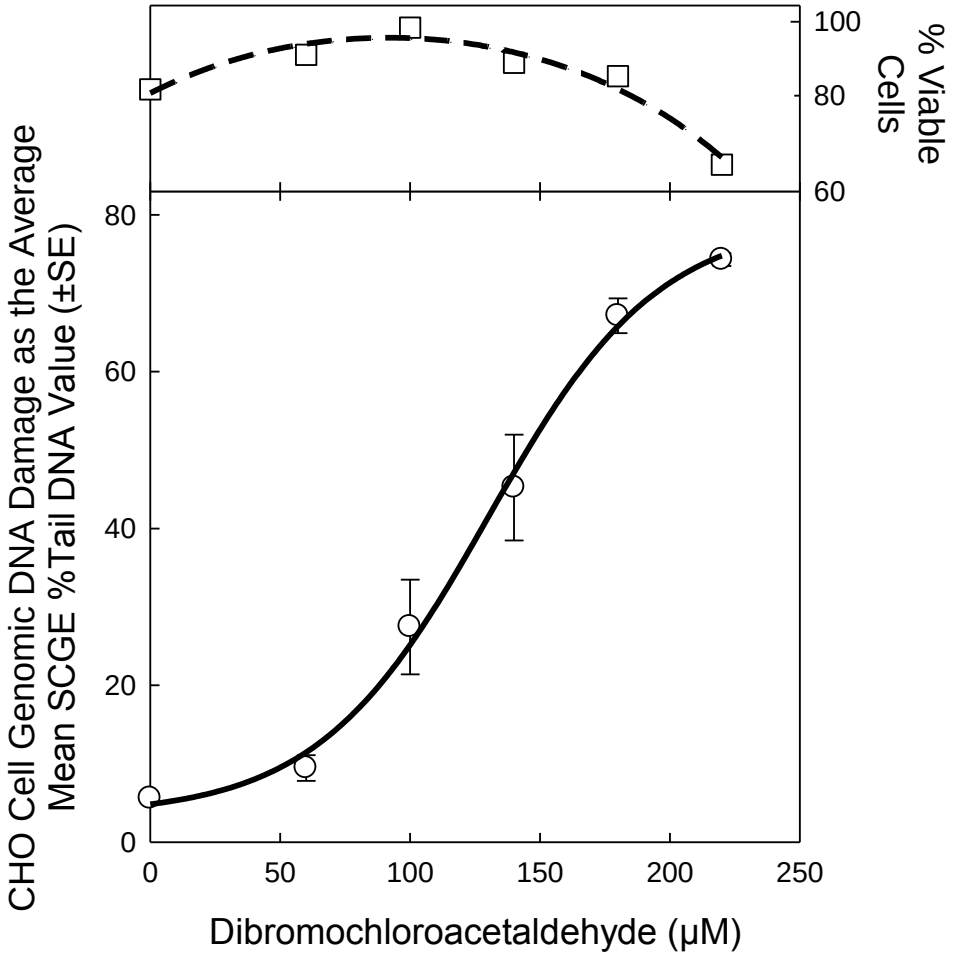


Figure 3.25. Dibromochloroacetaldehyde CHO cell genotoxicity.

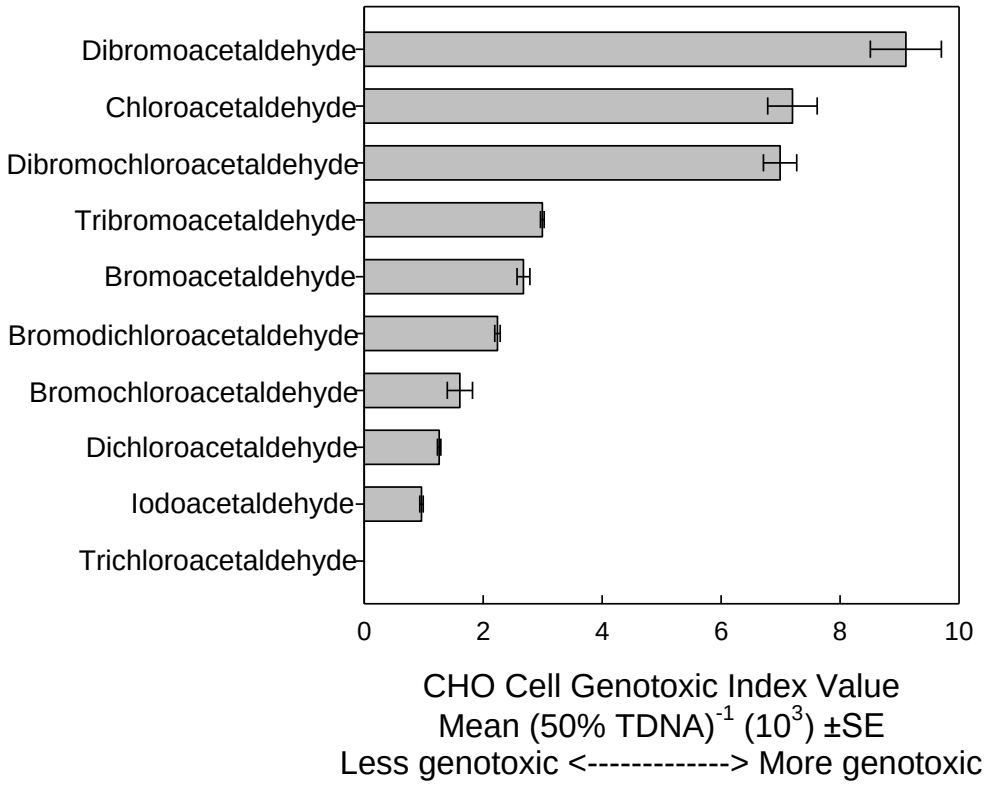


Figure 3.26. The mean bootstrap CHO Cell Genotoxic Index (GTI) values of ten haloacetaldehydes.

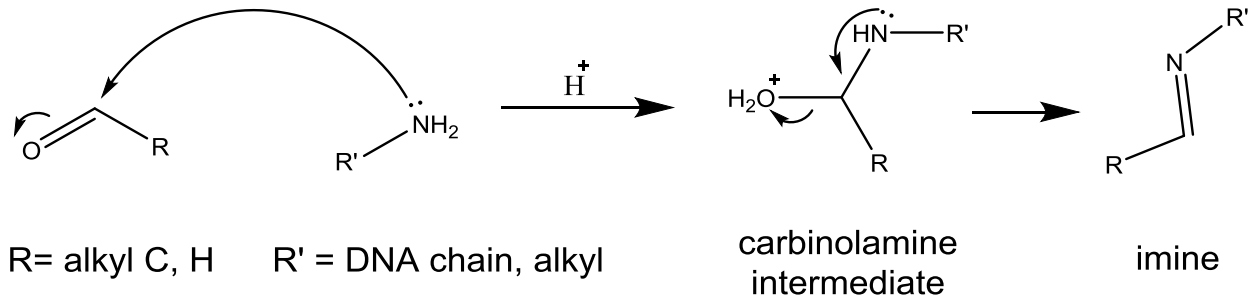
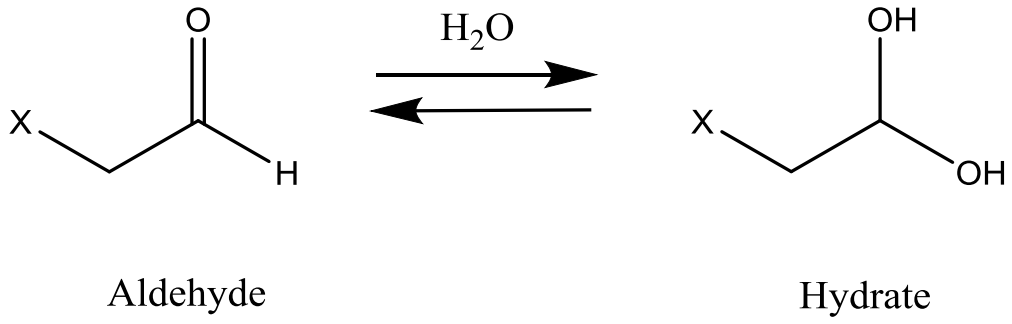


Figure 3.27. Schiff base formation by aliphatic aldehydes.



$$K_{Hydration} = [Hydrate] / [Aldehyde]$$

Figure 3.28. Equilibrium between aldehydes and hydrates.

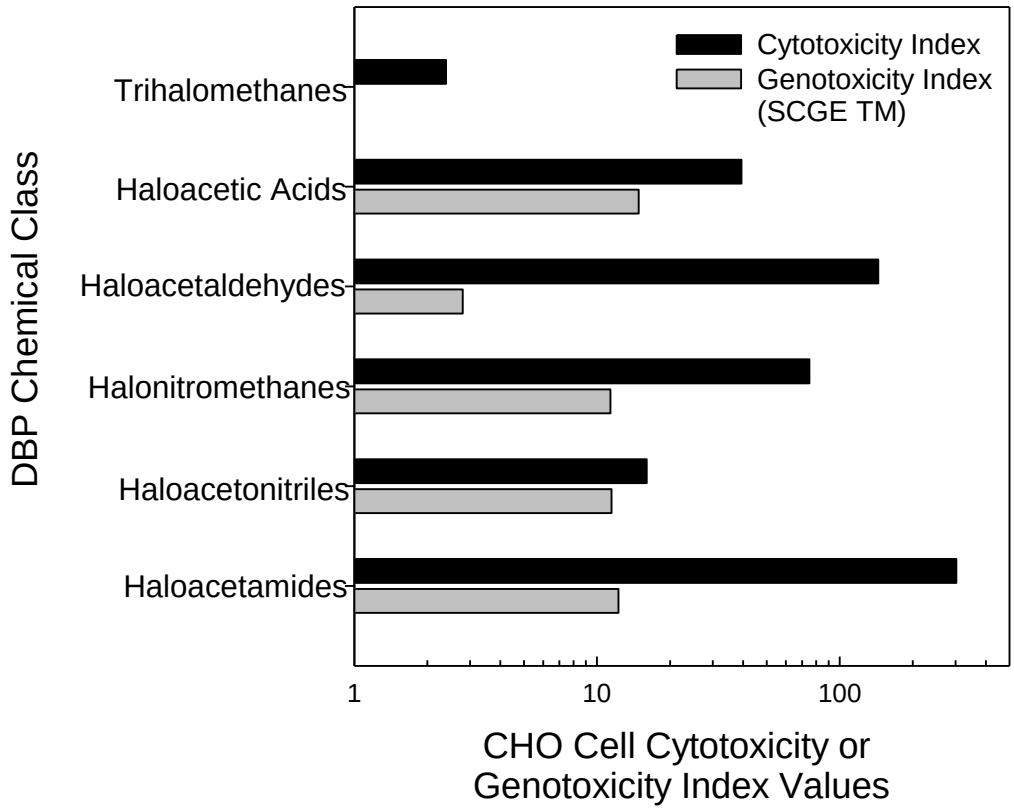


Figure 3.29. Comparisons of the CHO cell chronic cytotoxicity index values and acute genotoxicity index values of DBP chemical classes. The genotoxicity index was determined by calculating the mean SCGE genotoxic potency value which is defined by the SCGE tail moment from the individual compounds within a single class of DBP. (The SCGE tail moment is the integrated value of DNA density multiplied by the migration distance.)

CHAPTER 4

INVESTIGATE THE BIOLOGICAL MECHANISM INDUCED BY HALOACETIC ACID DISINFECTION BY-PRODUCTS AND THE DEVELOPMENT OF A SINGLE WELL MICROPLATE-BASED ATP-PROTEIN MEASUREMENT ASSAY

PREFACE

Part of this research was published: Dad, A.; Jeong, C. H.; Pals, J. A.; Wagner, E. D.; Plewa, M. J., Pyruvate remediation of cell stress and genotoxicity induced by haloacetic acid drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2013,** *54*, (8), 629-637. With permission from Wiley and Sons. A. Dad and C.H. Jeong were co-first authors of the paper.

4.1. INTRODUCTION

A preeminent public health accomplishment achieved during the last century was the disinfection of drinking water. Water treatment and distribution of disinfected water was an effective strategy in controlling waterborne diseases such as cholera, typhoid and dysentery [1]. However, disinfection by-products (DBPs) are inadvertently generated when chlorine or other disinfectants react with organic matter present in source water [1, 2]. Many DBPs are cytotoxic, genotoxic, mutagenic, carcinogenic and teratogenic [3]. Epidemiological studies demonstrated an association between lifetime exposures to DBPs and increased risk of bladder cancer [4-7],

colorectal cancer [8, 9] and skin cancer [10]. The U.S. Environmental Protection Agency (U.S. EPA) estimated that between 2 and 17% of bladder cancer cases in the U.S. may be induced by DBPs [11]. Epidemiological studies also demonstrated an association between disinfected drinking water and adverse pregnancy outcomes [12-14].

Currently, over 600 DBPs have been identified and the spectrum of DBP chemical classes are influenced by the source water, water contaminants and the disinfectant used [2, 15, 16]. In chlorinated water the second largest occurring DBP chemical class is the haloacetic acids (HAAs) [17]. The HAAs are the most highly regulated DBPs. The U.S. EPA regulates chloroacetic acid (CAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromoacetic acid (BAA), and dibromoacetic acid (DBAA) to a total maximum contaminant level of 60 µg/L [18]. At all water sites measured, the U.S. EPA Information Collection Rule recorded the mean and 90th percentile concentration for the 5 regulated HAAs (HAA5) as 23 µg/L and 47.5 µg/L, respectively [19].

HAAs are alkylating agents and follow S_N2 reactivity, which is primarily dependent on the α-carbon-halide (αC-X) bond length and the bond dissociation energy. The αC-X bond length follows the pattern of C-I > C-Br > C-Cl, which implies that the greater the bond length, the lower the dissociation energy required to react with the target molecule [20]. Cytotoxic and genotoxic potencies induced by the monohaloacetic acids (monoHAAs) expressed the pattern of iodoacetic acid (IAA) > BAA >> CAA, which highly correlated to the S_N2 reactivity, αC-X bond length and αC-X dissociation energy [20].

HAAs are cytotoxic and genotoxic in Chinese hamster ovary (CHO) cells [21] and non-transformed human cells [22]. They are mutagenic in *Salmonella typhimurium* and CHO cells [20, 23, 24] and toxic in a variety of other bioassays [3]. The monoHAAs modulated the gene expression pathways of ATM, MAPK, p53, BRCA1, BRCA2, and ATR, in non-transformed human FHs 74-Int cells originated from a female fetus 3-4 months into gestation [25]. These pathways are involved in stress response to DNA damage and regulate different stages in cell cycle progression or apoptosis [22, 26]. IAA induced malignant transformation in NIH/3T3 mouse embryonic fibroblast cells that progressed to highly aggressive fibrosarcomas when implanted in Balb/c nude mice [27]. Under *ex-vivo* conditions, monoHAAs were teratogenic and induced dysmorphogenesis in CD-1 mouse embryos and affected neural tube development, eye development and produced anomalies in heart development [28]. Gestational exposure of mixtures of HAA5 resulted in pregnancy loss and eye malformation in rats [29]. DCAA, DBAA, and bromochloroacetic acid (BCAA) altered intestinal microflora and metabolism in rats which could further affect bioactivations of promutagens or procarcinogens [30].

IAA induced toxicity in hippocampal neuronal cells by inhibiting the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which led to hypoglycemia and the generation of reactive oxygen species (ROS) [31]. Similar effects were induced by IAA in hippocampal astrocytes [32]. The direct role of monoHAA-mediated inhibition kinetics of GAPDH, their high correlation with many toxicity measurements and their impact on gene expression of human ROS regulation was recently published by our laboratory [33].

Our hypothesis is that GAPDH is inhibited by monoHAAs, which reduces the generation of pyruvate that is required for the tricarboxylic acid (TCA) cycle for the further production of ATP. We postulate that the unavailability of pyruvate causes mitochondrial stress leading to the generation of ROS and a reduction in cellular ATP levels, which may further induce cytotoxicity in HAA-treated cells. If this hypothesis is correct, then exogenous pyruvate supplementation in monoHAA-treated cells should restore cellular ATP levels. Along with the mechanistic study, we suggest that ATP measurement assay could be a novel bioassay which quantitatively measures the inhibition potency of a common cellular mechanism related to toxicity. However, to avoid the artifact such as different ATP levels caused by different cell viability of the cell suspensions, the experimental designs should be refined to include total protein analyses from the same suspension which the ATP levels are measured.

The objectives of this research were to: i) measure the impact of the monoHAAs on ATP levels with and without pyruvate supplementation, ii) develop a quantitative bioassay that will measure the reduction of ATP generation and the protein amount at non-cytotoxic levels of DBPs (pmol ATP/mg protein) from a same suspension, and analyze ten known HAAs with this new assay to test the sensitivity of this assay as a novel metric to compare ATP reduction potency of individual DBPs, and iii) analyze for correlation between the previously published toxicity data and the ATP reduction potency and determine the utility of the assay as an indicator of toxicological effects.

4.2. MATERIALS AND METHODS

4.2.1. Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). The sources and purities of the monoHAAs used in this research are listed in Table 4.1. The sources and purities of di- and triHAAs are listed in Table 4.2. Cell culture F12 medium and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. Pyruvic acid was purchased from Acros Organics (Fair Lawn, NJ). Cell Titer-Glo reagent was purchased from Promega (Madison, WI). The monoHAAs were dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C in sealed sterile glass vials (Sulpelco, Bellefonte PA). Pyruvate was dissolved directly either in F12 medium or Hank's balanced salt solution (HBSS) according to the experimental design. Individual HAA stock solutions in DMSO (1M) were diluted in F12 medium or HBSS depending upon the experimental design on the day of experiment.

4.2.2. Chinese Hamster Ovary Cells

CHO cell clone 11-4-8 was used [34]. Cells were grown in 100 mm glass petri plates with F12 medium containing 5% FBS, 1% glutamine and 1% antibiotic-antimycotic solution at 37 °C in a humidified atmosphere of 5% CO_2.

4.2.3. Pyruvate Supplementation: ATP Analysis

Cellular ATP levels were determined after exposure to monoHAAs alone, pyruvic acid alone or monoHAAs plus pyruvic acid with a TUNE-SpectraMax Paradigm® Multi-Mode Microplate Detection Platform (Sunnydale, CA) using Promega Cell Titer–Glo ATP reagents. The

day before ATP analysis, 3×10^4 CHO cells/well were cultured in a 96 well opaque microplate in 200 µL of F12 containing 5% FBS. The next day, the cells were washed with 100 µL of HBSS and treated with concentrations of the monoHAAs with and without pyruvic acid in 50 µL HBSS (with 1.3 mM $CaCl_2$ and 1.1 mM $MgSO_4$). The microplate was covered with AlumnaSeal and incubated for 4 h at 37°C, 5% CO_2. Each experiment contained a concurrent negative control, a bioluminescence background control, pyruvic acid (10 mM), the monoHAAs, monoHAAs plus pyruvic acid and an ATP standard curve. After incubation, the cells were washed with 100 µL of HBSS and supplemented with 10 mM pyruvate (for the pyruvate containing treatment groups), covered with AlumnaSeal and incubated for 30 min at 37°C, 5% CO_2 and then equilibrated to room temperature for 30 min. The ATP contents of the cells were measured according to the manufacturer's protocol using 50 µL of Cell Titer-Glo ATP reagents. Data were collected in an Excel spreadsheet and the pmol of ATP was calculated for each well.

4.2.4. Pyruvate Supplementation: Protein Determination

Parallel ATP and protein analyses were performed. The day before the experiment, 3×10^4 CHO cells/well were cultured in a 96 well flat bottom clear microplate in 200 µL of F12 containing 5% FBS. The next day, cells were treated with the HAAs with and without pyruvate as discussed above for the ATP analysis. After treatment, the cells were lysed by adding 25 µL of Solulyse® cell homogenizing solution (Genlantis, San Diego, CA). The microplate was covered with sterile AlumnaSeal and put on a rocker platform (at 37°C) shaken for 5 min after which the plate was rotated 90° and shaken for an additional 5 min. After cell lyses, 10 µL of the lysate from each well was transferred into a new microplate; 10 µL of an anti-foaming agent (Sigma

240, 0.01% v/v), 40 µL of Bradford solution (BioRad), and 140 µL of dH_2O for a final volume of 200 µL were added into each well. A BioRad protein (BSA) standard was prepared on the same microplate, using 0.68 µg/µL of a BSA standard solution. The contents of each well were carefully mixed and incubated at room temperature for 20 min. The absorbance was read at 595 nm using a SpectraMax Molecular Device plate reader. The data were collected in an Excel spreadsheet and the mg of protein was calculated for each well.

4.2.5. Single Well Approach: Cell Treatment

The day before the treatment, 3×10^4 CHO cells/well were cultured in a 96 well opaque microplate in 200 µL of F12 plus 5% FBS. The next day, the cells were washed with 100 µL of HBSS and treated with concentrations of the HAAs in 50 µL HBSS containing 1.3 mM $CaCl_2$ and 1.1 mM $MgSO_4$. Each experiment contained a concurrent negative control, a blank control, and two different concentrations of each HAA. The microplate was covered with AlumnaSeal and incubated for 3 h 30 min at 37°C, 5% CO_2, followed by additional 30 min incubation at room temperature (total 4 h treatment). After 4 h of treatment, the cells were washed with 100 µL of HBSS and were lysed by adding 25 µL of Solulyse® cell homogenizing solution (Genlantis, San Diego, CA). 10 µL of the lysate from each well were immediately transferred to a new 96 well clear microplate for protein measurement.

4.2.6. Single Well Approach: ATP Analysis

In the 96 well opaque microplate with the remaining 15 µL lysate, 10 µL of HBSS were added to each well. An ATP standard curve was set-up in the same plate. Cell Titer-Glo ATP reagents (25 µL) were added into each well, and the plate was incubated in the orbital shaker

for 2 min at 37°C, followed by an additional incubation at room temperature for 10 min. The ATP levels were measured using a bioluminescent agent with a TUNE-SpectraMax Paradigm® Multi-Mode Microplate Detection Platform. Data were collected in an Excel spreadsheet and the pmol of ATP per 25 µL lysate was calculated for each well.

4.2.7. Single Well Approach: Protein Determination

In the 96 well clear microplate containing 10 µL of lysate, 10 µL of an anti-foaming agent, Antifoam 204 (0.01% v/v, Sigma-Aldrich Co.) were added. A protein standard curve was conducted in the same microplate with a BSA standard solution. 40 µL of Bradford solution (BioRad) and 140 µL of dH_2O were added and each well was mixed gently by using a multi-pipet. The plate was incubated for 20 min at room temperature before measurement. The absorbance was read at 595 nm using a SpectraMax Molecular Device plate reader. Data were collected in an Excel spreadsheet and mg of protein per 25 µL lysate was calculated for each well.

4.2.8. Statistical Analysis

A Pearson's Product Moment correlation test was conducted to investigate the correlations of ATP alteration potency with HAA physiochemical parameters and various toxicological parameters including cytotoxicity, genotoxicity, mutagenicity, and teratogenicity as well as the GAPDH inhibition kinetics in CHO cells. The number of groups analyzed per experiment was two (HAA-treated and its independent concurrent control). A *t*-test was performed to examine the statistical significance of the generation of the bioluminescence units in the HAA-treated cells compared to its concurrent negative control.

4.3. RESULTS

4.3.1. Depletion of ATP by MonoHAAs

CHO cells were treated with 25 and 40 µM IAA, 60 µM BAA and 6 mM CAA for 4 h in HBSS. The concentrations of each monoHAA were previously determined to be genotoxic in CHO cells without acute cytotoxicity [35]. The cellular ATP levels (as the average bioluminescence unit) for monoHAA-treated cells were significantly reduced as compared to the negative control. The ATP levels with CAA-treated cells were approximately the same as the blank (Figure 4.1). By observation, we did not detect CHO cell cytotoxicity after 4 h.

4.3.2. Pyruvate Supplementation and ATP Levels in MonoHAA-treated Cells

We determined the minimum concentration of each monoHAA in HBSS (with 1.3 mM $CaCl_2$ and 1.1 mM $MgSO_4$) that induced a significant reduction in cellular ATP levels. The lowest non-cytotoxic concentrations of IAA, BAA and CAA that induced a significant reduction in ATP levels as compared to each concurrent negative control were 3 µM, 6 µM, and 1 mM (Figure 4.2). Using these concentrations of IAA, BAA and CAA, we conducted parallel experiments to determine the absolute amount of ATP and the total protein content of each cell suspension within each treatment group. This experimental design allowed us to determine concentrations of ATP and protein for each CHO cell suspension for a precise measurement of ATP depletion and restoration. The concentration of ATP as pmol/mg protein for each monoHAA treatment group is presented in Table 4.3. Figure 4.3 illustrates cellular ATP levels (pmol ATP/mg protein) normalized as 100% for each concurrent negative control for each monoHAA treatment group. A reduction in ATP levels was observed with exposures to 3 µM IAA, 6 µM BAA and 1 mM CAA

with ATP levels of 18.7%, 48.2% and 50.8% of the concurrent negative controls, respectively. When monoHAA-treated cells were simultaneously treated with 10 mM pyruvate, a significant recovery of cellular ATP levels was measured. For IAA, ATP levels increased from 18.7% to 45.0% of the concurrent negative control. Similar responses were observed for BAA (48.2% to 122.1%) and CAA (50.8% to 80.2%) (Figure 4.3). The CHO cells treated with IAA had the greatest reduction in cellular ATP levels (0.54%) per pmol IAA. For the BAA and CAA treatment groups, the reduction in cellular ATP levels per pmol monoHAA was 0.15% for BAA and 0.001% for CAA (Table 4.3).

4.3.3. Single Well Approach: Alteration of ATP Levels in HAA-treated Cells

CHO cells were treated with HAA for 4 h in HBSS (with 1.3 mM $CaCl_2$ and 1.1 mM $MgSO_4$) with two concentrations of each HAA. Treated concentrations were previously determined to be genotoxic in CHO cells without acute cytotoxicity [35]. The minimum concentration of each HAA that induced a significant reduction in cellular ATP levels as compared to each concurrent negative control was used for further analysis (Table 4.6 and Figure 4.5). Effects of the HAAs on ATP levels compared to its concurrent negative control in CHO cells with t-test analysis are presented in Table 4.6. The cellular ATP levels for monoHAA-treated cells were significantly reduced, but were not concentration-dependent (Figure 4.4). Figure 4.5 illustrates cellular ATP levels (pmol ATP/mg protein) normalized as 100% for each concurrent negative control for HAA treatment group. All ATP levels except TCAA showed a statistically significant alteration of celluler ATP levels. Interestingly, while all mono- and diHAAs showed a reduction in ATP levels, three triHAAs showed an increase of ATP levels. The % alteration of cellular ATP per pmol HAA

in CHO cells and other toxicological parameters of HAAs are presented in Table 4.7. We calculated the CHO cell ATP alteration potency index value (|%Alteration per pmol HAA| $\times 10^3$) for each HAA (Table 4.7).

To investigate correlations between CHO cell ATP alteration potency and other HAA toxicological parameters, we applied a Pearson's Product Moment statistical test (Table 4.8) [36]. The cytotoxic potency significantly correlated with monoHAA %ATP alteration potency (r = 0.997; $P \leq 0.05$) and all HAA %ATP alteration potency (r = 0.997; $P \leq 0.001$). The genotoxic potency significantly correlated with all HAA %ATP alteration potency (r = 0.961; $P \leq 0.001$). The cytotoxic potency with diHAA %ATP alteration potency, and the genotoxic potency with monoHAA %ATP alteration potency showed good, but not statistically significant correlations (r = 0.992; P = 0.081 and r = 0.950; P = 0.202).

4.4. DISCUSSION

4.4.1. Depletion of ATP Levels and the Effect of Pyruvate Supplementation in MonoHAA-Treated Cells

All three monoHAAs induced ATP depletion in CHO cells, and when they were simultaneously treated with 10 mM pyruvate, a significant recovery of cellular ATP levels was measured. The monoHAA-mediated ATP depletion followed a rank order of IAA > BAA >> CAA. This pattern and magnitude of ATP depletion directly correlated with the αC-X bond length and relative alkylation potential of each monoHAA and was inversely correlated with the αC-X bond dissociation energy (Table 4.4). ATP depletion was highly correlated with the inhibition kinetics

of GAPDH [33] (Table 4.6). A conserved cysteine residue in the active site of GAPDH is central to its glycolytic function [37-39]. This cysteine serves as a nucleophile in the first catalytic step in the conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate [40]. The αC of each monoHAA is a primary alkyl halide and an electrophile due to electron withdrawal from the carbon by the halogen substituent. IAA, BAA and CAA inhibit GAPDH when the α-carbon undergoes an S_N2 reaction with the nucleophilic thiol group on the catalytic cysteine residue. This results in a carboxymethylated cysteine which irreversibly inhibits the catalytic function of the enzyme.

Our results suggest that a major pathway in the toxic mode of action by the monoHAAs is the irreversible inhibition of GAPDH and the subsequent reduction of cellular ATP levels induced by a blockage in the generation of pyruvate from glucose. Previous studies demonstrated that IAA blocked glycolysis by inhibiting GAPDH that led to neurotoxicity [31, 32, 41], ROS generation [31, 41], ATP depletion [31, 32] and disruption of intracellular Ca^{2+} homeostasis [32, 42]. Of importance is that ATP depletion by the monoHAAs is highly correlated with diverse measurements of toxicity including cytotoxicity, genotoxicity, mutagenicity and teratogenicity published in the literature over the past 17 years (Table 4.5).

Overall, our suggested molecular mechanism is that the monoHAAs inhibit GAPDH by alkylating the thiol group at the GAPDH active site which leads to glycolytic ATP depletion and blocks the production of pyruvate that is a mitochondrial substrate for the TCA cycle [39]. Pyruvate starvation enhances mitochondrial stress, disrupts the TCA cycle and affects the generation of reducing power (NADH and $FADH_2$) within mitochondria during the TCA cycle.

This deficiency in reducing power disturbs normal oxidative phosphorylation, which eventually generates ROS, depletes mitochondrial ATP and increases cytosolic Ca^{2+} concentration [31, 40, 43, 44]. This proposed mechanism is supported in the pattern of toxicological response of IAA > BAA > CAA and that IAA-induced mutagenicity in *Salmonella typhimurium* and genotoxicity in CHO cells was repressed by the antioxidants catalase and butylated hydroxyanisole [45]. We believe that ATP depletion and ROS generation are the principal forcing mechanisms for monoHAA-mediated toxicity [33, 46, 47].

4.4.2. Alteration of ATP Levels in HAA-treated Cells Determined by a Novel Microplate-based Single Well ATP-protein Measurement Assay

The cellular ATP levels for monoHAA-treated cells were significantly reduced, but were not concentration-dependent (Figure 4.4). This result suggests that the new single well assay is less sensitive than the parallel assay used of the pyruvate supplementation experiment which showed a concentration-dependent manner (Figure 4.2). The blank bioluminescence signal of the single well experiment was an order of magnitude lower than the blank signal of the parallel experiment. Therefore, the low sensitivity of the single well assay may be due to the smaller amount of Cell Titer-Glo ATP reagents used of the measurement compared to the parallel assay which leads to a lower resolving power. However, it should be emphasized that the new assay was able to obtain statistically significant changes in bioluminescence signals. The fact that the measurement of protein was performed within the same cell suspension used for the ATP level measurement strengthens the accuracy of the calculated ATP alteration potency index value. Since the single well assay only needs half amount of the testing

compound compared to the parallel assay, once the single well assay is refined and further optimized, it may be useful for testing the toxicity of limited amount of samples such as complex DBP mixtures.

An interesting trend was found in the ATP levels in terms of the number of halogens attached (Table 4.6 and Figures 4.5-4.6). MonoHAAs showed the greatest reduction in ATP levels, whereas diHAAs showed a moderate reduction with higher concentration ranges. TriHAAs induced significant increase in ATP levels except for TCAA. The average ATP levels as the % of negative control was 40.9% for monoHAAs, 72.9% for diHAAs, and 120% for triHAAs (Figure 4.6). These results were unexpected because from a previous experiment in our laboratory, all ten HAAs induced an inhibition of GAPDH activity. The increased ATP level for triHAAs indicates that HAAs may be involved in other ATP regulation pathways in addition to GAPDH activity.

Whitehouse *et al.* reported that TCAA inhibits pyruvate dehydrogenase kinase (PDK) and increased the proportion of active pyruvate dehydrogenase (PDH) in the pig heart [48]. PDH is an enzyme in the pyruvate dehydrogenase complex (PDC) which converts pyruvate into acetyl-CoA. This decarboxylation step is known to be the rate-limiting step for the pyruvate PDC machinery. Since acetyl-CoA is an essential substrate for TCA cycle, PDH activity is important in linking the glycolysis pathway to the TCA cycle, and ATP production. Therefore, it is possible that the increase of ATP levels were induced by triHAAs because the dominant molecular pathway was the activation of PDH instead of GAPDH inhibition. DCAA also inhibited the PDK at approximately the same concentrations as for TCAA in the same study. Later, Kline *et al.* found

that DCAA activates myocardial PDH and increase myocardial tissue content of ATP [49]. This indicates that diHAAs can also activate PDH and increase ATP production. Since DCAA showed the lowest ATP alteration potency index value (index value = 0.087, Table 4.7), it is possible that diHAAs have similar extent of activities in both GAPDH inhibition and PDH activation to compensate the overall ATP level as a result. However, further investigation is needed to confirm this hypothesis.

One other possible mechanism related to ATP production is the pyruvate transport pathway into mitochondria. In eukaryotic cells, the glycolysis occurs in the cytosol, and the TCA cycle occurs in the mitochondria matrix. Thus, pyruvate generated through glycolysis needs to get transported into the mitochondria matrix for ATP production. In a study investigating the kinetics of pyruvate transport into mitochondria, CAA and DCAA appeared to be a substrate for the mitochondrial pyruvate carrier, while TCAA did not, which indicates that mono- and diHAAs may interfere with pyruvate carrier to alter the ATP production [50]. However, whether these results are due to different mechanisms that involving cellular energy homeostasis are unclear.

4.5. CONCLUSION

This study extended our research on the molecular mechanism(s) of toxicity of these important DBPs by investigating the impact of monoHAAs on cellular ATP levels and the attenuation by pyruvate supplementation. This study also provided a platform for the development of a novel bioassay, which quantitatively measures the toxicity of DBPs at molecular cellular levels. An alteration in cellular ATP homeostasis at non-cytotoxic

concentration may be an important measure of toxic action. Additional studies are needed to validate the utility of the assay, and we will further determine if PDH is stimulated by the di- and triHAAs.

References

1. Cutler, D.; Miller, G., The role of public health improvements in health advances: the twentieth-century United States. *Demography* **2005,** *42*, (1), 1-22.
2. Richardson, S. D., Disinfection by-products: formation and occurrence in drinking water. In *Encyclopedia of Environmental Health*, Nriagu, J. O., Ed. Elsevier: Burlington, 2011; Vol. 1, pp 110-136.
3. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636*, (1-3), 178-242.
4. Costet, N.; Villanueva, C. M.; Jaakkola, J. J.; Kogevinas, M.; Cantor, K. P.; King, W. D.; Lynch, C. F.; Nieuwenhuijsen, M. J.; Cordier, S., Water disinfection by-products and bladder cancer: is there a European specificity? A pooled and meta-analysis of European case-control studies. *Occup. Environ. Med.* **2011,** *68*, (5), 379-385.
5. Kogevinas, M., Epidemiological approaches in the investigation of environmental causes of cancer: the case of dioxins and water disinfection by-products. *Environ. Health* **2011,** *10 Suppl 1*, S3.
6. Villanueva, C. M.; Cantor, K. P.; Cordier, S.; Jaakkola, J. J.; King, W. D.; Lynch, C. F.; Porru, S.; Kogevinas, M., Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* **2004,** *15*, (3), 357-367.
7. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.
8. King, W. D.; Marrett, L. D.; Woolcott, C. G., Case-control study of colon and rectal cancers and chlorination by-products in treated water. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **2000,** *9*, (8), 813-818.
9. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39*, (3), 733-745.
10. Karagas, M. R.; Villanueva, C. M.; Nieuwenhuijsen, M.; Weisel, C. P.; Cantor, K. P.; Kogevinas, M., Disinfection byproducts in drinking water and skin cancer? A hypothesis. *Cancer Causes Control* **2008,** *19*, (5), 547-548.
11. U. S. Environmental Protection Agency *Quantification of Cancer Risk from Exposure of Chlorinated Drinking Water*; Office of Water, Office of Science and Technology, Health and Ecological Criteria Division: Washington, D.C., 1998.
12. Costet, N.; Garlantezec, R.; Monfort, C.; Rouget, F.; Gagniere, B.; Chevrier, C.; Cordier, S., Environmental and urinary markers of prenatal exposure to drinking water disinfection by-products, fetal growth, and duration of gestation in the PELAGIE birth cohort (Brittany, France, 2002-2006). *Am. J. Epidemiol.* **2012,** *175*, (4), 263-275.

13. Jeong, C. H.; Wagner, E. D.; Siebert, V. R.; Anduri, S.; Richardson, S. D.; Daiber, E. J.; McKague, A. B.; Kogevinas, M.; Villanueva, C. M.; Goslan, E. H.; Luo, W.; Isabelle, L. M.; Pankow, J. F.; Grazuleviciene, R.; Cordier, S.; Edwards, S. C.; Righi, E.; Nieuwenhuijsen, M. J.; Plewa, M. J., The occurrence and toxicity of disinfection byproducts in European drinking waters in relation with the HIWATE epidemiology study. *Envion. Sci. Technol.* **2012,** *46*, (21), 12120-12128.
14. Righi, E.; Bechtold, P.; Tortorici, D.; Lauriola, P.; Calzolari, E.; Astolfi, G.; Nieuwenhuijsen, M. J.; Fantuzzi, G.; Aggazzotti, G., Trihalomethanes, chlorite, chlorate in drinking water and risk of congenital anomalies: A population-based case-control study in Northern Italy. *Environ. Res.* **2012,** *116*, 66-73.
15. Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Res.* **2007,** *41*, (8), 1667-1678.
16. Zhang, X.; Echigo, S.; Minear, R. A.; Plewa, M. J., Characterization and comparison of disinfection by-products of four major disinfectants. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 299-314.
17. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., The occurrence of a new generation of disinfection by-products. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-7185.
18. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 disinfectants and disinfection byproducts rule. *Fed. Reg.* **2006,** *71*, (2), 387-493.
19. McGuire, M. J.; McLain, J. L.; Obolensky, A., *Information Collection Rute Data Analysis*. AwwaRF and AWWA: Denver, CO, 2002.
20. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D., Jr.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38*, (18), 4713-4722.
21. Plewa, M. J.; Simmons, J. E.; Richardson, S. D.; Wagner, E. D., Mammalian cell cytotoxicity and genotoxicity of the haloacetic acids, a major class of drinking water disinfection by-products. *Environ. Mol. Mutagen.* **2010,** *51*, (8-9), 871-878.
22. Attene-Ramos, M. S.; Wagner, E. D.; Plewa, M. J., Comparative human cell toxicogenomic analysis of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2010,** *44*, (19), 7206-7212.
23. Kargalioglu, Y.; McMillan, B. J.; Minear, R. A.; Plewa, M. J., Analysis of the cytotoxicity and mutagenicity of drinking water disinfection by-products in *Salmonella typhimurium*. *Teratog. Carcinog. Mutagen.* **2002,** *22*, (2), 113-128.
24. Zhang, S. H.; Miao, D. Y.; Liu, A. L.; Zhang, L.; Wei, W.; Xie, H.; Lu, W. Q., Assessment of the cytotoxicity and genotoxicity of haloacetic acids using microplate-based cytotoxicity test and CHO/HGPRT gene mutation assay. *Mutat. Res.* **2010,** *703*, (2), 174-179.
25. Smith, H. S., In vitro properties of epithelial cell lines established from human carcinomas and nonmalignant tissue. *J. Natl. Cancer Inst.* **1979,** *62*, (2), 225-230.
26. Muellner, M. G.; Attene-Ramos, M. S.; Hudson, M. E.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis of bromoacetic acid: a regulated drinking water disinfection by-product. *Environ. Mol. Mutagen.* **2010,** *51*, 205-214.

27. Wei, X.; Wang, S.; Zheng, W.; Wang, X.; Liu, X.; Jiang, S.; He, G.; Zheng, Y.; Qu, W., Tumorigenicity of drinking water disinfection byproduct iodoacetic acid in NIH3T3 cells. *Envion. Sci. Technol.* **2013,** *47*, (11), 5913-5920.
28. Hunter, E. S.; Rogers, E. H.; Schmid, J. E.; Richard, A., Comparative effects of haloacetic acids in whole embryo culture. *Teratology* **1996,** *54*, (2), 57-64.
29. Narotsky, M. G.; Best, D. S.; McDonald, A.; Godin, E. A.; Hunter, E. S., 3rd; Simmons, J. E., Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. *Reprod. Toxicol.* **2011,** *31*, (1), 59-65.
30. George, S. E.; Nelson, G. M.; Swank, A. E.; Brooks, L. R.; Bailey, K.; George, M.; DeAngelo, A., The disinfection by-products dichloro-, dibromo-, and bromochloroacetic acid impact intestinal microflora and metabolism in Fischer 344 rats upon exposure in drinking water. *Toxicol. Sci.* **2000,** *56*, (2), 282-289.
31. Hernandez-Fonseca, K.; Cardenas-Rodriguez, N.; Pedraza-Chaverri, J.; Massieu, L., Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons. *J. Neurosci. Res.* **2008,** *86*, (8), 1768-1780.
32. Kahlert, S.; Reiser, G., Requirement of glycolytic and mitochondrial energy supply for loading of $Ca^{(2+)}$ stores and InsP(3)-mediated $Ca^{(2+)}$ signaling in rat hippocampus astrocytes. *J. Neurosci. Res.* **2000,** *61*, (4), 409-420.
33. Pals, J. A.; Ang, J. K.; Wagner, E. D.; Plewa, M. J., Biological mechanism for the toxicity of haloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2011,** *45*, (13), 5791-5797.
34. Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J., Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998,** *32*, (4), 360-368.
35. Komaki, Y.; Pals, J.; Wagner, E. D.; Marinas, B. J.; Plewa, M. J., Comparative DNA damage and repair kinetics study in mammalian cells by chloro-, bromo,- and iodoacetic acid. In *Environmental Mutagen Society 40th Annual Meeting*, Wiley-Blackwell: St. Louis, MO, 2009.
36. Box, G. E. P.; Hunter, W. G.; Hunter, J. S., *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*. Wiley & Sons Inc.: New York, NY., 1978.
37. Carneiro, A. S.; Lameira, J.; Alves, C. N., A theoretical study of the molecular mechanism of the GAPDH Trypanosoma cruzi enzyme involving iodoacetate inhibitor. *Chem. Phys. Lett.* **2011,** *514*, (4-6), 336-340.
38. Guido, R. V. C.; Balliano, T. L.; Andricopulo, A. D.; Oliva, G., Kinetic and Crystallographic Studies on Glyceraldehyde-3-Phosphate Dehydrogenase from Trypanosoma cruzi in Complex with Iodoacetate. *Lett Drug Des Discov* **2009,** *6*, (3), 210-214.
39. Tristan, C.; Shahani, N.; Sedlak, T. W.; Sawa, A., The diverse functions of GAPDH: Views from different subcellular compartments. *Cell. Signal.* **2011,** *23*, (2), 317-323.

40. Nakajima, H.; Amano, W.; Fujita, A.; Fukuhara, A.; Azuma, Y. T.; Hata, F.; Inui, T.; Takeuchi, T., The active site cysteine of the proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase is essential in oxidative stress-induced aggregation and cell death. *J. Biol. Chem.* **2007,** *282*, (36), 26562-26574.
41. Matthews, R. T.; Ferrante, R. J.; Jenkins, B. G.; Browne, S. E.; Goetz, K.; Berger, S.; Chen, I. Y.; Beal, M. F., Iodoacetate produces striatal excitotoxic lesions. *J. Neurochem.* **1997,** *69*, (1), 285-289.
42. Chinopoulos, C.; Adam-Vizi, V., Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. *FEBS J.* **2006,** *273*, (3), 433-450.
43. Csordas, G.; Hajnoczky, G., SR/ER-mitochondrial local communication: calcium and ROS. *Biochim. Biophys. Acta.* **2009,** *1787*, (11), 1352-1362.
44. Peng, T. I.; Jou, M. J., Oxidative stress caused by mitochondrial calcium overload. *Ann. N.Y. Acad. Sci.* **2010,** *1201*, 183-188.
45. Cemeli, E.; Wagner, E. D.; Anderson, D.; Richardson, S. D.; Plewa, M. J., Modulation of the cytotoxicity and genotoxicity of the drinking water disinfection byproduct Iodoacetic acid by suppressors of oxidative stress. *Environ. Sci. Technol.* **2006,** *40*, (6), 1878-1883.
46. Pals, J.; Attene-Ramos, M. S.; Xia, M.; Wagner, E. D.; Plewa, M. J., Human cell toxicogenomic analysis linking reactive oxygen species to the toxicity of monohaloacetic acid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2013,** *47*, (21), 12514-12523.
47. Schlisser, A. E.; Yan, J.; Hales, B. F., Teratogen-induced oxidative stress targets glyceraldehyde-3-phosphate dehydrogenase in the organogenesis stage mouse embryo. *Toxicol. Sci.* **2010,** *118*, (2), 686-695.
48. Whitehouse, S.; Cooper, R. H.; Randle, P. J., Mechanism of activation of pyruvate dehydrogenase by dichloroacetate and other halogenated carboxylic acids. *Biochem. J.* **1974,** *141*, (3), 761-774.
49. Kline, J. A.; Maiorano, P. C.; Schroeder, J. D.; Grattan, R. M.; Vary, T. C.; Watts, J. A., Activation of pyruvate dehydrogenase improves heart function and metabolism after hemorrhagic shock. *J. Mol. Cell Cardiol.* **1997,** *29*, (9), 2465-2474.
50. Halestrap, A. P., The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. *Biochem. J.* **1975,** *148*, (1), 85-96.
51. Loudon, G. M., *Organic Chemistry*. 3rd ed.; Benjamin/Cummings Publ. Co.: Redwood, CA, 1995.
52. Richard, A. M.; Hunter, E. S., 3rd, Quantitative structure-activity relationships for the developmental toxicity of haloacetic acids in mammalian whole embryo culture. *Teratology* **1996,** *53*, (6), 352-360.

TABLES AND FIGURES

Table 4.1. Monohaloacetic acid characteristics, sources and purities.

HAA [a]	CASN	MW (g/mol)	C−X [b]	Bond Length (Å) [c]	Dissociation Energy (kcal/mol) [d]	Source	Purity (%)
IAA	64-69-7	185.95	C−I	2.14	57.4	Sigma-Aldrich	>99
BAA	79-08-3	138.95	C−Br	1.93	65.9	Fluka	>99
CAA	79-11-8	94.50	C−Cl	1.77	78.5	Fluka	>99

[a] Abbreviations: HAA, haloacetic acids; IAA, iodoacetic acid; BAA, bromoacetic acid; CAA, chloroacetic acid. [b] α-Carbon-halogen bond. [c] C−X bond length summarized from [51].[d] C−X bond dissociation energy summarized from [51].

Table 4.2. Di- and trihaloacetic acids characteristics, sources and purities.

HAA [a]	CASN	MW (g/mol)	Source	Purity (%)
DCAA	79-43-6	128.94	Fluka	>99
DBAA	631-64-1	217.86	Fluka	>97
BCAA	5589-96-8	173.39	U.S. EPA	>98
TCAA	76-03-9	163.39	Fluka	>99
TBAA	75-96-7	296.76	Fluka	>98
DBCAA	5278-95-5	252.30	Cerilliant	>98
BDCAA	71133-14-7	207.84	Radian International	>98

[a] Abbreviations: HAA, haloacetic acids; DCAA, dichloroacetic acid; DBAA, dibromoacetic acid; BCAA, bromochloroacetic acid; TCAA, trichloroacetic acid; TBAA, tribromoacetic acid; DBCAA, dibromochloroacetic acid; BDCAA, bromodichloroacetic acid.

Table .4.3. Effects of the monoHAAs on ATP levels in CHO cells with and without pyruvate supplementation.

HAA Group	Treatment Conditions	pmol ATP/mg Protein Mean Value ± SE	% Reduction per pmol HAA
IAA	Negative Control (IAA)	29788 ± 3197	NA
	10 mM Pyruvate	29476 ± 3575	NA
	3 µM IAA	5557 ± 274	0.54229
	3 µM IAA + Pyruvate	13411 ± 1105	NA
BAA	Negative Control (BAA)	18225 ± 1503	NA
	10 mM Pyruvate	29297 ± 2782	NA
	6 µM BAA	8786 ± 897	0.15136
	6 µM BAA + Pyruvate	24338 ± 2487	NA
CAA	Negative Control (CAA)	6814 ± 755	NA
	10 mM Pyruvate	23464 ± 2278	NA
	1 mM CAA	3463 ± 419	0.00098
	1 mM CAA + Pyruvate	5465 ± 589	NA

NA, not applicable.

Table 4.4. Pearson Product Moment Correlation analyses of HAA physiochemical parameters and the percent reduction of cellular ATP per pmol HAA in CHO cells.

Physicochemical Parameters	ELUMO (*r*) [a]	αC–X Bond Length (*r*) [b]	αC–X Dissociation Energy (*r*) [b]	Relative Alkylation Potential (S_N2) (*r*) [b]
ATP % Reduction per pmol HAA [c]	−0.986	0.985	−0.935	0.999
ELUMO		−1.000	0.981	−0.980
C–X Bond Length			−0.982	0.979
C–X Dissociation Energy				−0.922

[a] Calculated ELUMO (energy of the lowest unoccupied molecular orbital) summarized from [52]. [b] Summarized from [51]. [c] Calculated from data presented in this paper.

Table 4.5. Pearson Product Moment Correlation analyses of HAA toxicological parameters and the percent reduction of cellular ATP per pmol HAA in CHO cells.

HAA Toxicological Parameters	CHO cell cytotoxic index (*r*)	CHO cell genotoxic index (*r*)	FHs cell genotoxic index (*r*)	*Salmonella* cytotoxic index (*r*)	*Salmonella* mutagenic potency (*r*)	CHO cell mutagenicity index (*r*)	Mouse teratogenicity (*r*)	GAPDH inhibition (*r*)
ATP % reduction per pmol HAA [a]	1.00	0.968	0.992	0.991	0.993	0.965	0.999	−0.986
CHO cell cytotoxic index [b]		0.976	0.996	0.995	0.996	0.956	0.998	−0.990
CHO cell genotoxic index [c]			0.992	0.993	0.991	0.868	0.959	−0.997
FHs cell genotoxic index [c]				1.00	1.00	0.925	0.987	−0.999
Salmonella cytotoxic index [d]					1.00	0.921	0.986	−0.999
Salmonella mutagenic potency [d]						0.926	0.988	−0.999
CHO cell mutagenicity index [e]							0.974	−0.906
Mouse teratogenicity [f]								−0.979
GAPDH inhibition kinetics [g]								1.00

[a] Calculated from data presented in this paper. [b] Derived as the reciprocal of the LC_{50} concentration (× constant to generate whole numbers), data from [21]. [c] Derived as the reciprocal of the SCGE genotoxic potency value (× constant to generate whole numbers), data from [21, 33]. [d] Data from [20, 23]. [e] Data from [24]. [f] Data from [28]. [g] The average rate of GAPDH inhibition per µM monoHAA concentration derived from the slope of the inhibition curves for each monoHAA [33].

Table 4.6. Effects of the HAAs on ATP levels compared to its concurrent negative control in CHO cells and t-test statistical analysis calculated with the LUM as the % of negative control.

HAA Group	Number of Replicates (N)	Average LUM (RLU ± SE)	Average LUM as the % of control (± SE)	*t*	df	*P*
CAA control	32	4142.4 ± 185.6	100.00 ± 4.48	10.820	62	<0.001
CAA 700 µM	32	1386.9 ± 174.4	33.48 ± 4.21			
BAA control	23	5195.4 ± 222.9	100.00 ± 4.29	10.943	45	<0.001
BAA 15 µM	24	2585.0 ± 94.5	49.76 ± 1.82			
IAA control	32	4856.3 ± 309.2	100.00 ± 6.37	8.529	62	<0.001
IAA 4 µM	32	1917.0 ± 152.2	39.47 ± 3.13			
DCAA control	24	4708.8 ± 90.4	100.00 ± 1.92	3.133	44	0.003
DCAA 2000 µM	22	4296.0 ± 96.0	103.71 ± 2.04			
DBAA control	24	5270.1 ± 93.7	100.00 ± 1.78	14.270	46	<0.001
DBAA 700 µM	24	3707.1 ± 56.6	70.34 ± 1.07			
BCAA control	34	6309.8 ± 177.4	100.00 ± 2.81	9.545	66	<0.001
BCAA 700 µM	34	4147.8 ± 136.2	66.16 ± 2.16			
TCAA control	31	4760.9 ± 226.3	100.00 ± 4.75	-0.749	61	0.456
TCAA 2000 µM	32	4969.6 ± 164.5	104.38 ± 3.46			
TBAA control	24	4220.3 ± 208.5	100.00 ± 4.94	-3.830	46	<0.001
TBAA 1000 µM	24	5168.1 ± 133.3	122.46 ± 3.16			
BDCAA control	30	4852.3 ± 82.8	100.00 ± 1.71	-5.453	60	<0.001
BDCAA 2000 µM	32	5883.6 ± 165.8	121.25 ± 3.42			
DBCAA control	32	4771.7 ± 191.4	100.00 ± 4.01	-4.473	62	<0.001
DBCAA 1000 µM	32	6044.0 ± 210.4	126.66 ± 4.41			

Abbreviations: HAA, haloacetic acid; CAA, chloroacetic acid; BAA, bromoacetic acid; IAA, iodoacetic acid; DCAA, dichloroacetic acid; DBAA, dibromoacetic acid; BCAA, bromochloroacetic acid; TCAA, trichloroacetic acid; TBAA, tribromoacetic acid; BDCAA, bromodichloroacetic acid; DBCAA, dibormochloroacetic acid; LUM, luminescence; RLU, relative light unit; SE, standard error; df, degrees of freedom.

Table 4.7. Toxicological parameters of HAAs and the percent alteration of cellular ATP per pmol HAA in CHO cells.

HAA Group	Treated Concentration (μM)	% Alteration per pmol HAA	CHO Cell Cytotoxic Potency Index [a,d]	CHO Cell Genotoxic Potency Index [b,d]	CHO Cell ATP Alteration Potency Index [c]
CAA	700	1.90×10^{-3}	12.3	24.4	1.90
BAA	15	6.7×10^{-2}	1000	588	67.0
IAA	4	3.03×10^{-1}	3450	1150	303
DCAA	2000	8.66×10^{-5}	1.27	NA	0.087
DBAA	700	1.11×10^{-3}	16.9	5.56	1.11
BCAA	700	9.67×10^{-4}	12.8	3.33	0.97
TCAA	2000	-9.20×10^{-5}	4.17	NA	0.092
TBAA	1000	-4.49×10^{-4}	118	4.00	0.45
BDCAA	2000	-2.13×10^{-4}	14.5	NA	0.21
DBCAA	1000	-5.86×10^{-4}	50.0	0.714	0.59

Abbreviations: HAA, haloacetic acid; CAA, chloroacetic acid; BAA, bromoacetic acid; IAA, iodoacetic acid; DCAA, dichloroacetic acid; DBAA, dibromoacetic acid; BCAA, bromochloroacetic acid; TCAA, trichloroacetic acid; TBAA, tribromoacetic acid; BDCAA, bromodichloroacetic acid; DBCAA, dibormochloroacetic acid.

[a] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1}\times10^{2}$) for each HAA. [b] The CHO cell genotoxic potency index value corresponds to (SCGE $GP^{-1}\times10^{-2}$), where SCGE GP value is the HAA concentration that was calculated, using regression analysis, at the midpoint of the curve within the concentration range that expressed above 70% cell viability. [c] The CHO cell ATP alteration potency index value corresponds to ($|\%\text{Alteration per pmol HAA}|\times10^{3}$) for each HAA. [d] Data from [21].

Table 4.8. Pearson Product Moment Correlation analyses of HAA toxicological parameters and the % ATP alteration potency of HAA groups categorized by the number of halogens in the α-carbon.

HAA Group	CHO Cell Cytotoxic Potency Index [a,d] ($LC_{50}^{-1}\times10^{2}$)	CHO Cell Genotoxic Potency Index [b,d] (SCGE $GP^{-1}\times10^{-2}$)	CHO Cell Cytotoxic Potency vs CHO Cell Genotoxic Potency
MonoHAA CHO Cell ATP Alteration Potency Index [c]	$r = 0.997$ $P \leq 0.05$	$r = 0.950$ $P = 0.202$	$r = 0.971$ $P = 0.154$
DiHAA CHO Cell ATP Alteration Potency Index [c]	$r = 0.992$ $P = 0.0813$	NA	NA
TriHAA CHO Cell ATP Alteration Potency Index [c]	$r = 0.676$ $P = 0.324$	NA	NA
All HAA CHO Cell ATP Alteration Potency Index [c]	$r = 0.997$ $P \leq 0.001$	$r = 0.961$ $P \leq 0.001$	$r = 0.976$ $P \leq 0.001$

Abbreviations: HAA, haloacetic acid.

[a] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1}\times10^{2}$) for each HAA. [b] The CHO cell genotoxic potency index value corresponds to (SCGE $GP^{-1}\times10^{-2}$), where SCGE GP value is the HAA concentration that was calculated, using regression analysis, at the midpoint of the curve within the concentration range that expressed above 70% cell viability. [c] The CHO cell ATP alteration potency index value corresponds to ($|\%\text{Alteration per pmol HAA}|\times10^{3}$) for each HAA. [d] Data from [21].

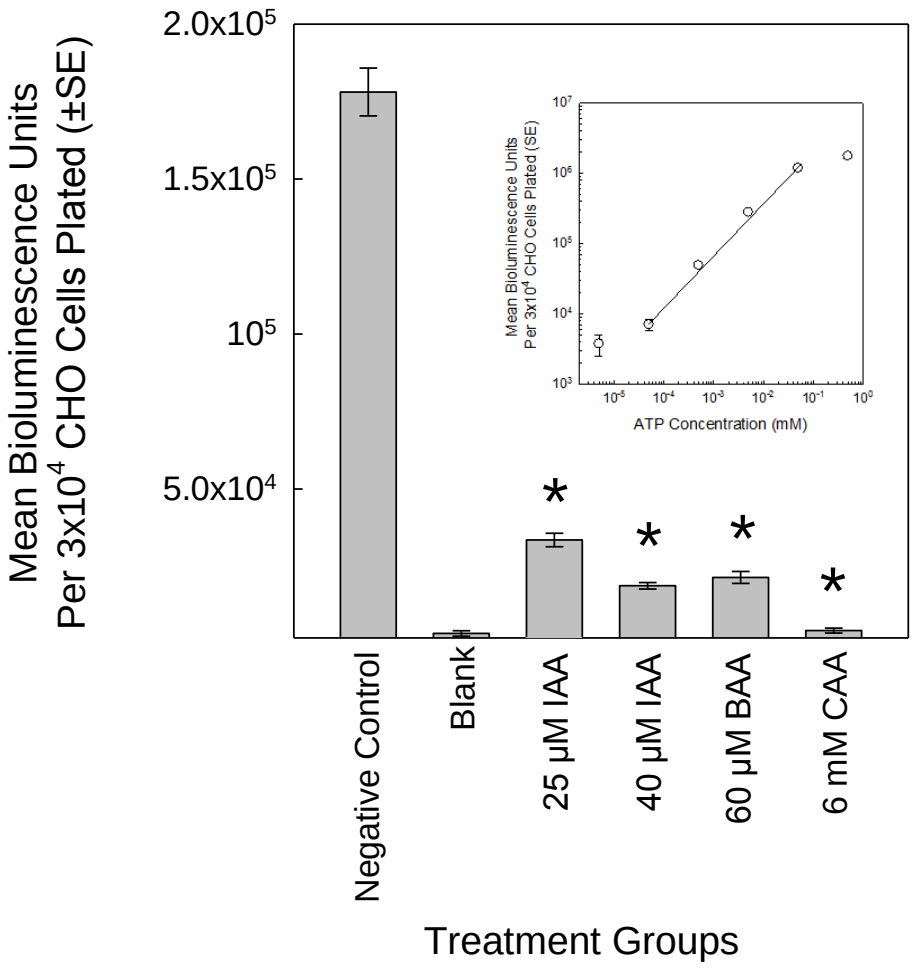


Figure 4.1. Impact of monoHAA exposure on the cellular ATP levels as measured using relative bioluminescence units. The * indicates a significant difference from the negative control. The insert is a ATP standard curve.

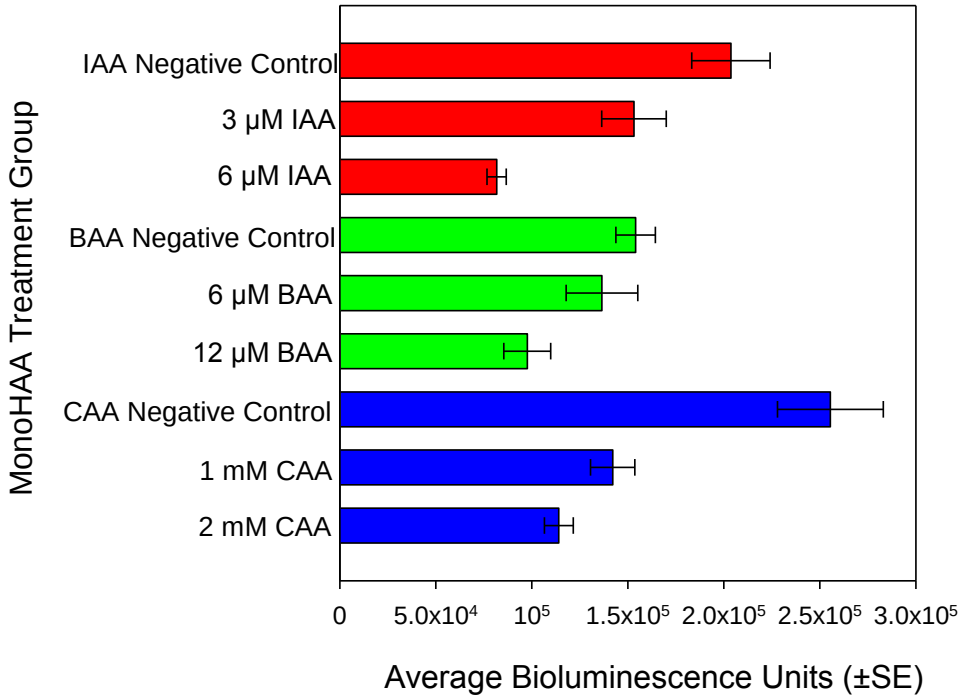


Figure 4.2. Reduction of ATP levels measured as bioluminescence units in CHO cells after exposure to monoHAAs. All monoHAA treatments induced a significant reduction in ATP levels as compared to the concurrent negative controls.

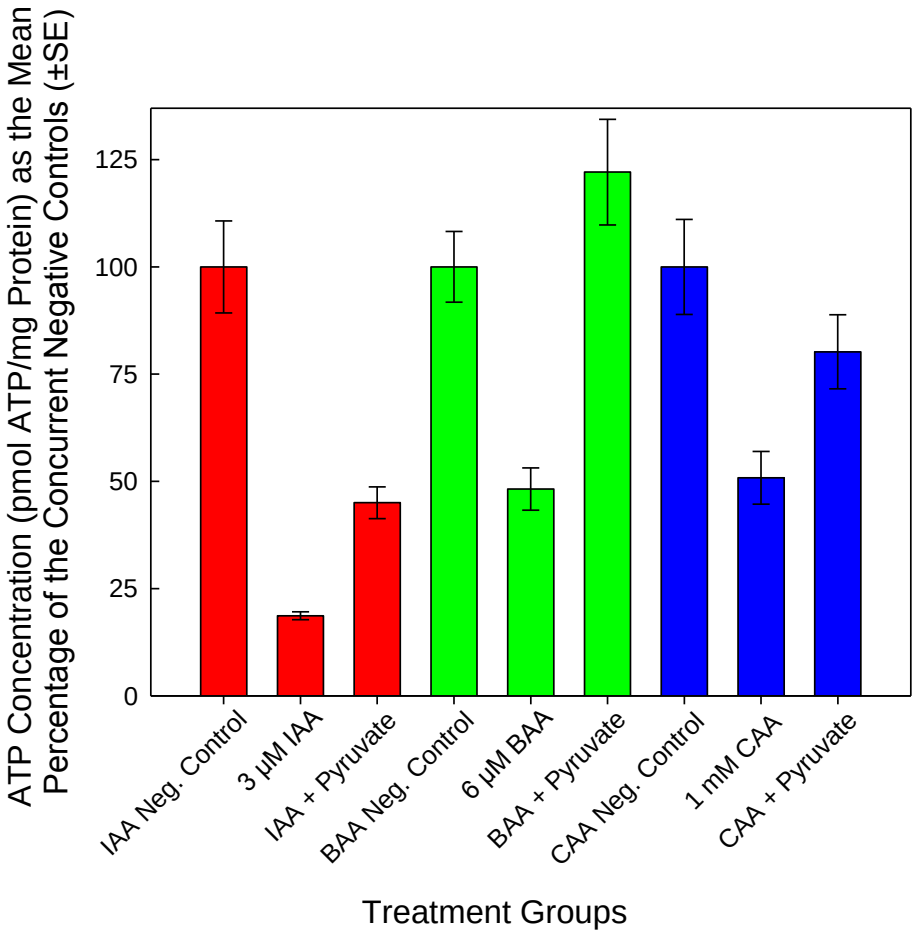


Figure 4.3. Recovery of ATP levels in monoHAA-treated cells after pyruvate supplementation.

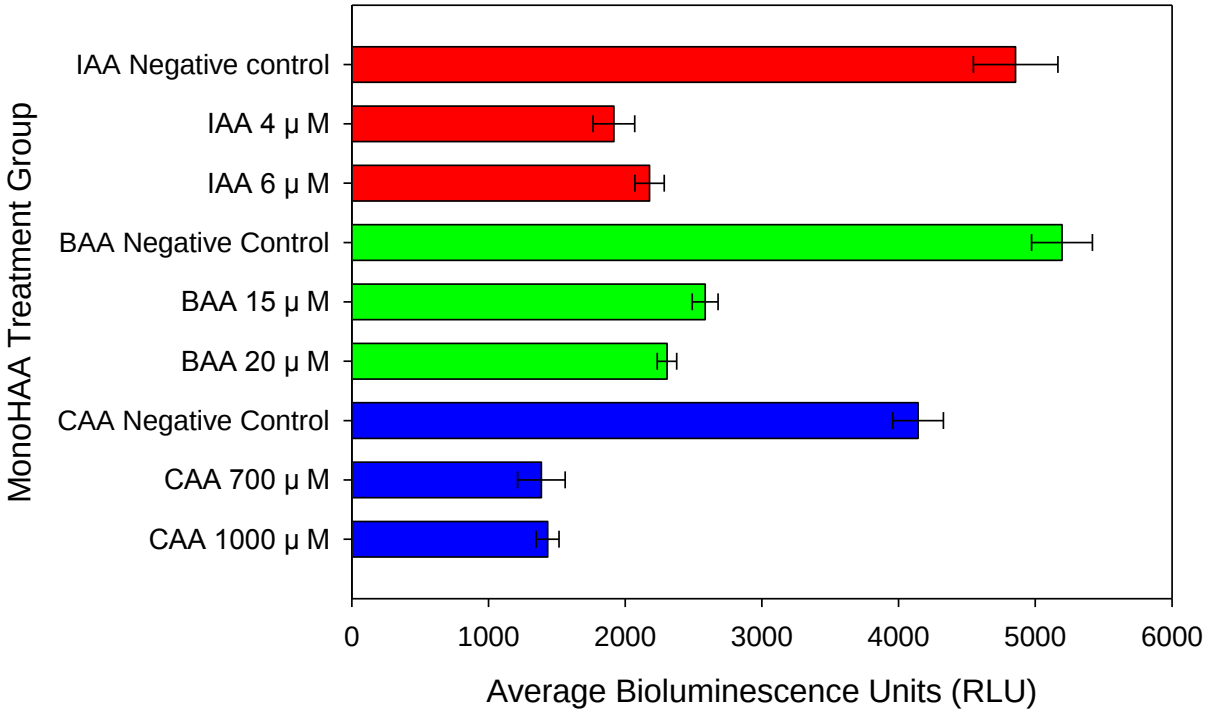


Figure 4.4. Reduction of ATP levels measured as bioluminescence units in CHO cells after exposure to monoHAAs using a single well approach bioassay. All monoHAA treatments induced a significant reduction in ATP levels as compared to the concurrent negative controls.

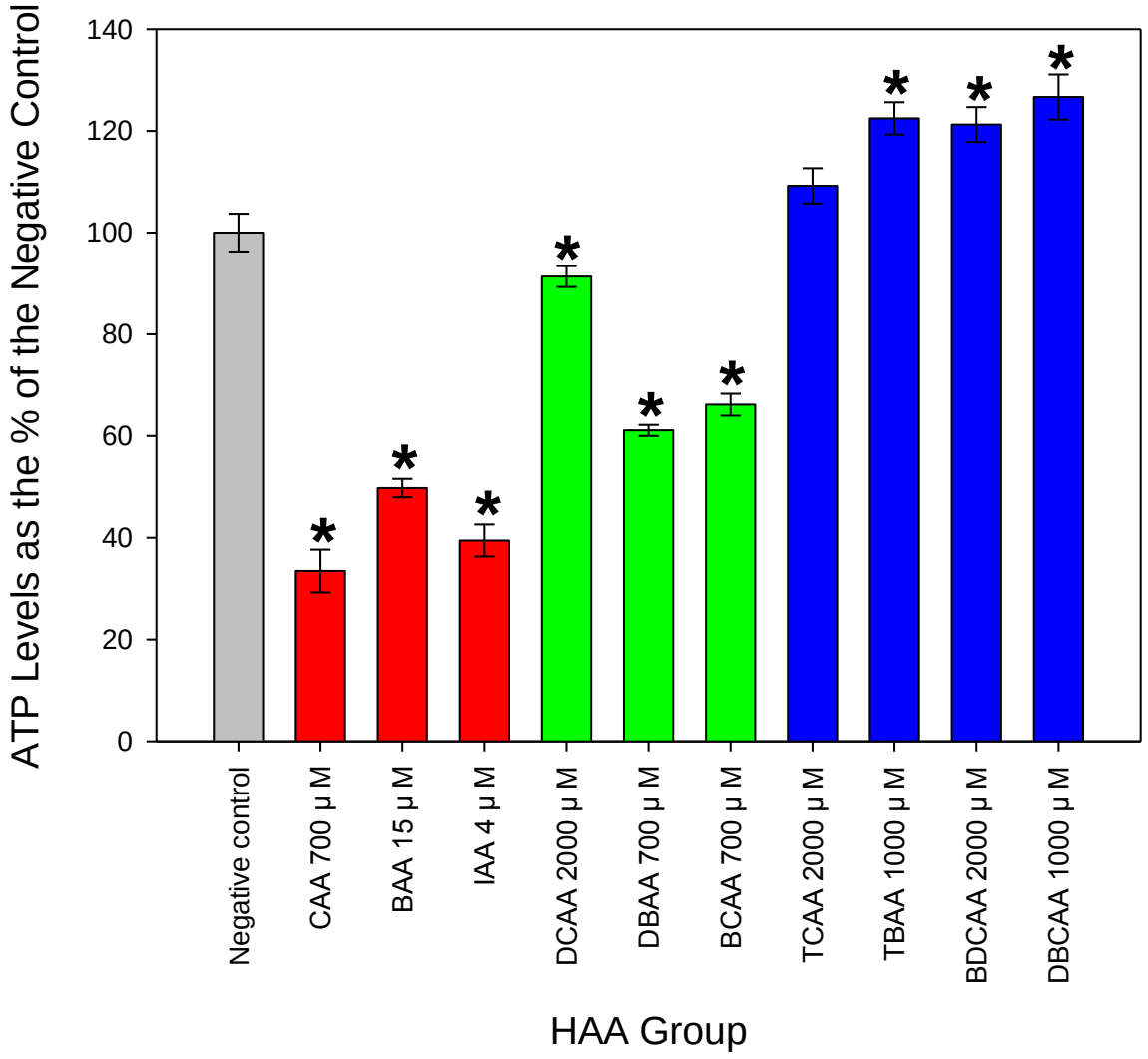


Figure 4.5. Impacts of HAA exposure on the cellular ATP levels (pmol ATP/mg protein). ATP levels are normalized as 100% for each concurrent negative control for each HAA treatment group. Standard error (SE) used for the negative control is the average of all individual negative control SEs. The * indicates a statistically significant difference from their concurrent negative control (t-test, See Table 4.6).

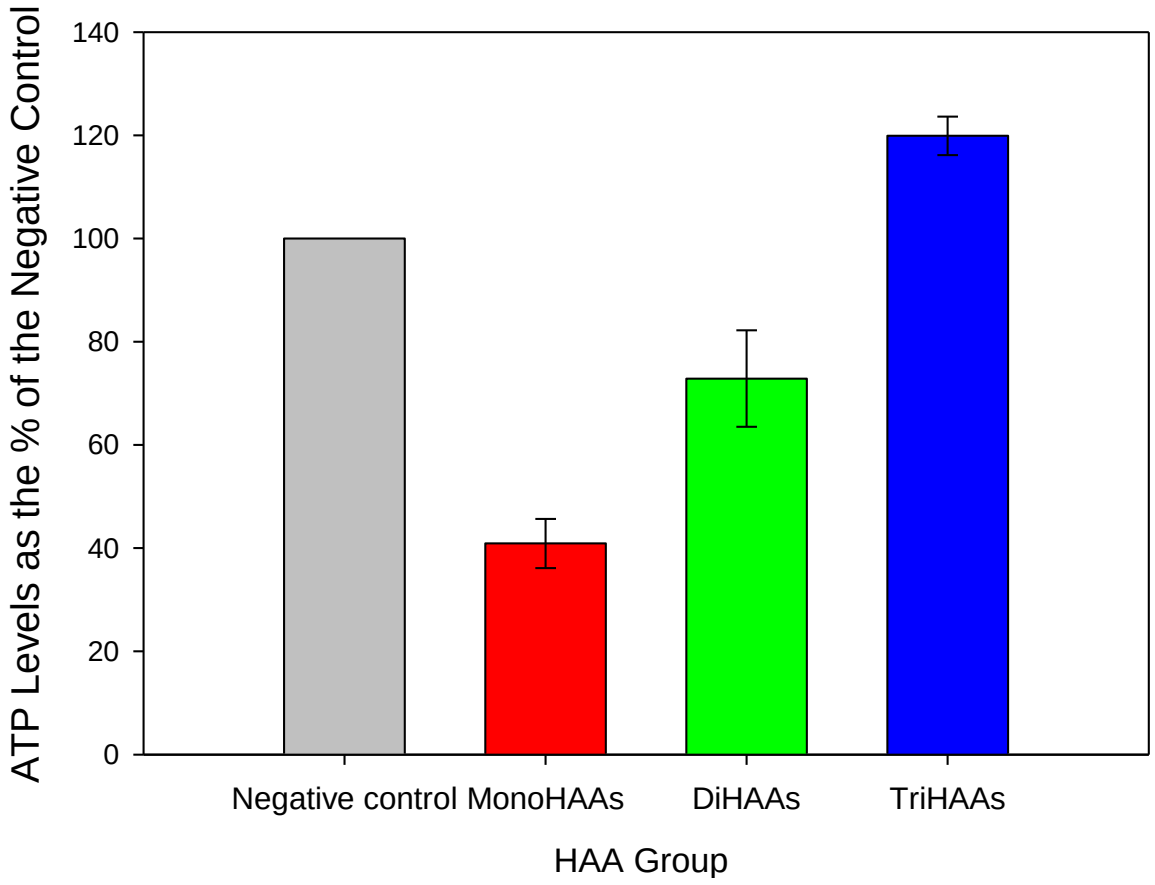


Figure 4.6. Impacts of numbers of halogens attached to HAA on the cellular ATP levels (pmol ATP/mg protein). ATP levels were normalized as 100% for each concurrent negative control for each HAA treatment group. Individual ATP % alteration values were grouped by the number of halogen attached and averaged.

CHAPTER 5

THE OCCURRENCE AND TOXICITY OF DISINFECTION BY-PRODUCTS IN EUROPEAN DRINKING WATERS IN RELATION WITH THE HIWATE EPIDEMIOLOGY STUDY

PREFACE

This research was published in: Jeong, C. H.; Wagner, E. D.; Siebert, V. R.; Anduri, S.; Richardson, S. D.; Daiber, E. J.; McKague, A. B.; Kogevinas, M.; Villanueva, C. M.; Goslan, E. H.; Luo, W.; Isabelle, L. M.; Pankow, J. F.; Grazuleviciene, R.; Cordier, S.; Edwards, S. C.; Righi, E.; Nieuwenhuijsen, M. J.; Plewa, M. J., The occurrence and toxicity of disinfection byproducts in European drinking waters in relation with the HIWATE epidemiology study. *Envion. Sci. Technol.* **2012,** *46*, (21), 12120-12128. With permission from American Chemical Society. The analytical chemistry work was conducted by Dr. S. Richardson (University of South Carolina).

5.1. INTRODUCTION

The introduction of water disinfection greatly reduced the incidence of waterborne infectious diseases [1]. Although chlorine is the most common disinfectant, alternatives include ozone, chloramines, chlorine dioxide, and UV radiation [2-4]. An unintended consequence of disinfection is the formation of drinking water disinfection by-products (DBPs) from the reaction between organic and inorganic materials in the water and disinfectants. Chemical

classes of DBPs include halomethanes, haloacetic acids (HAAs), and nitrogen-containing DBPs (N-DBPs); to date, more than 600 DBPs have been identified in drinking water [5, 6]. The spectrum of DBP generation depends on the source water, pH, temperature, disinfection type and processes [6-9]. Less than 20 DBPs are currently regulated in the United States and in other countries [5, 10].

Previous epidemiological studies reported associations between DBPs in chlorinated water and increased cancer risk [11-15] as well as DBPs and adverse pregnancy outcomes including spontaneous abortion, low birth weight (LBW), small-for-gestational-age (SGA), still birth, and preterm delivery [16-19]. HAAs were teratogenic in mice embryos [20]; mixtures of trihalomethanes (THMs) and HAAs were teratogenic in rats [21].

In 2006, the European Union (EU) established the project HIWATE (**H**ealth **I**mpacts of long-term exposure to disinfection byproducts in drinking **WATE**r) to investigate potential human health risks associated with long-term exposure to DBPs [22]. Pregnancy cohorts (N ≈23,000) were included from France, Lithuania, Spain, Italy, and the United Kingdom (Figure 5.1). These locations encompassed a variety of disinfectants and treatments including chlorine, ozone, chlorine dioxide, and desalination with reverse osmosis (Table 5.1). Metrics for adverse pregnancy outcomes were LBW, SGA, preterm delivery, fetal growth restriction (FGR), and parameters derived from ultrasound medical diagnosis.

This project represents the first systematic analysis combining DBP analytical chemistry and in vitro mammalian cell toxicology with adverse pregnancy outcomes. Our objectives were to (i) obtain disinfected drinking water from HIWATE cities, extract and concentrate the organic

fraction and chemically analyze for DBPs, (ii) determine the relative chronic cytotoxicity and acute genotoxicity in mammalian cells for each HIWATE sample, and (iii) analyze for correlations between the toxicity data and the occurrence and concentrations of DBPs.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and Reagents

General reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) and Fisher Scientific Co. (Itasca, IL). Media and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. (Itasca, IL). Chemical standards were purchased from Sigma-Aldrich, ChemService (West Chester, PA), Orchid Cellmark (Westminster, BC, Canada), and TCI America (Waltham, MA) at the highest level of purity.

5.2.2. Sample Preparation

Drinking water samples (20 L) were collected from 11 different distribution systems from 7 cities within 5 European countries, where an epidemiologic study of reproductive outcomes was being conducted. Samples were collected from March-June 2010 using 2L Teflon bottles (headspace-free) and were commercially shipped in coolers with icepacks to the U.S. Environmental Protection Agency (U.S. EPA) laboratory in Athens, GA. Water samples were extracted immediately upon arrival using XAD resins [23]. The final extract (2 mL in ethyl acetate) was equally divided for GC/MS analysis and genotoxicity/cytotoxicity analysis. For toxicity analyses the solvent ethyl acetate was evaporated with a stream of dry N_2 and

exchanged to dimethylsulfoxide (DMSO) resulting in a $10^5\times$ concentration. These samples were stored in glass Supelco 1-mL Micro Reaction Vessels (No. 27036) at −20 °C.

5.2.3. Broad-Screen GC/MS Analyses

Half of the extract was derivatized with diazomethane [24] to identify halo-acids (through their corresponding methyl esters), while the other half was analyzed directly for other DBPs. Comprehensive gas chromatography/mass spectrometer (GC/MS) analyses were performed on a high-resolution magnetic sector mass spectrometer (Autospec, Waters, Inc.) in electron ionization mode, equipped with an Agilent model 6890 gas chromatograph and operated at an accelerating voltage of 8 kV and source temperature of 200 °C, in both low-resolution (1000) and high-resolution (10,000) modes. Injections of 1 µL of the extracts were introduced via a split/splitless injector (in splitless mode) onto a GC column (ZB-5, 30 m X 0.25 mm ID, 0.25 µm film thickness, Phenomenex (Torrance, CA). The GC temperature program consisted of an initial temperature of 35 °C (4 min) followed by an increase at 9 °C/min to 285 °C (held for 30 min). Transfer lines were held at 280 °C and the injection port at 250 °C. To prevent decomposition of THMs, separate analyses were made with an injection port temperature of 180 °C [25]. For analysis of data by the Massworks expert system [26], extracts were analyzed in the continuum mode at 1000 resolution.

Mass spectra of unknown compounds in the drinking water extracts were subjected to library database searching (National Institute of Standards and Technology and Wiley databases). For DBPs not present in either database, high-resolution-MS and Massworks software (Cerno Bioscience, Norwalk, CT) were used to provide empirical formulas for

molecular ions and fragments. Mass spectra were also interpreted extensively to provide tentative structural identifications. When possible, pure standards were obtained to confirm identifications through a match of GC retention times and mass spectra.

5.2.4. GC X GC-TOF-MS Measurements

GC X GC-time-of-flight (TOF)-MS measurements were conducted using a Leco Pegasus 4D GC X GC-TOF mass spectrometer (Leco Corp., St. Joseph, Michigan). The extracts (1 µL) were introduced via a split/splitless injector (in splitless mode). A DB-VRX (45 m, 0.25 mm i.d., 1.4 µm film thickness, Agilent, Santa Clara CA) served as the primary column and a Stabilwax (1.5 m, 0.25 mm i.d., 0.25 µm film thickness, Restek, Bellefonte, PA) as the secondary column. The primary GC oven program consisted of an initial temperature of 45 °C (3 min), an increase at 10 °C/min to 145 °C (3 min), an increase at 5oC/min to 240 °C, and final hold of 20 min. The secondary GC oven was 13 °C above the primary GC oven. The modulator offset was 20 °C above to the primary GC oven. The modulation period was 7 s with 1.5 s hot pulse. The transfer line and source temperature were maintained at 248 °C and 200 °C, respectively. The MS data were acquired from m/z 35 to 500 at rate of 150 spectra/s in electron ionization mode.

5.2.5. Quantitative Chemical Analyses

THMs (chloroform, bromodichloromethane, dibromochloromethane, and bromoform), haloacetonitriles (dichloroacetonitrile, bromochloroacetonitrile, dibromoacetonitrile, and trichloroacetonitrile), haloketones (1,1-dichloro- and 1,1,1-trichloropropanone), trichloroacetaldehyde (chloral hydrate), and trichloronitromethane (chloropicrin) were extracted using a modified form of U.S. EPA Method 551.1 [27]. HAAs (chloro-, bromo-,

dichloro-, trichloro-, bromochloro-, dibromo-, bromodichloro-, dibromochloro-, and tribromoacetic acid) were analyzed using a modified form of U.S. EPA Method 552.3 [28]. The limit of detection for each DBP was 1 μg/L, with the exception of chloroacetic acid (detection limit was 2 μg/L).

5.2.6. Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8 was used for the biological assays [29-31]. CHO cells were maintained on glass culture plates in Ham's F12 medium containing 5% fetal bovine serum (FBS), 1% antibiotics (100 U/mL sodium penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

5.2.7. CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density as a function of the organic extract concentration over a period of approximately 3 cell divisions (72 h). Chronic cytotoxicity to CHO cells was measured using an assay we previously developed for the analysis of DBPs [32]. Flat-bottom, tissue culture 96-well microplates were employed; 4 replicate wells were prepared for each concentration of a specific organic extract for each HIWATE water sample. Eight wells were reserved for the blank control consisting of 200 μL of F12 medium + 5% fetal bovine serum (FBS). The negative control consisted of 8 wells containing 100 μL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 μL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known concentration of an HIWATE water sample organic extract for a total of 200 μL. To prevent cross-over contamination between wells due to

volatilization of the organic extract, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells, and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 10 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed in tap water, inverted and tapped dry upon paper towels, and 50 µL of dimethyl sulfoxide/methanol (3:1 v/v) was added to each well for 10 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet on a microcomputer connected to the microplate reader. The blank-corrected absorbance value of the negative control (cells with medium only) was set at 100%. The absorbance for each treatment group well was converted into a percentage of the negative control. For each organic extract concentration, 4-8 replicate wells were analyzed per experiment, and the experiments were repeated 2-3 times.

5.2.8. Single Cell Gel Electrophoresis Assay

Single cell gel electrophoresis (SCGE) is a molecular genetic assay that quantitatively measures the level of genomic DNA damage induced in individual nuclei of treated cells [33-35]. We employed the microplate SCGE method [36]. The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 µL of F12 + 5% FBS and incubated. The next day, the cells were washed with Hank's balanced salt solution (HBSS) and treated with a series of concentrations of an organic extract from the HIWATE water samples in F12 medium without FBS in a total volume of 25 µL for 4 h at 37°C, 5% CO_2. The wells were covered with sterile

AlumnaSeal™. After incubation, the cells were washed 2× with HBSS and harvested with 50 µL of 0.01% trypsin + 53 µM EDTA. The trypsin was inactivated with 70 µL of F12 + FBS. Acute cytotoxicity was measured from a 10 µL aliquot of cell suspension mixed with 10 µL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS) [37]. SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The cellular membranes were removed by an overnight immersion in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) at 4°C. The microgels were placed in an alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were neutralized with Tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis of the HIWATE samples, the microgels were hydrated in cold water for 30 min and stained with 65 µL of ethidium bromide (20 µg/mL) for 3 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, 2 microgels were prepared per treatment group. Randomly chosen nuclei (25 per microgel) were analyzed using a charged coupled device camera. A computerized image analysis system (Comet IV, Perspective Instruments, Ltd, Suffolk, UK) was employed to determine the SCGE %Tail DNA value of the nuclei as indices of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet

for subsequent statistical analysis. Within each experiment, a negative control, a positive control (3.8 mM ethylmethanesulfonate), and 9 concentrations of an organic extract from HIWATE water samples were analyzed concurrently. The experiments were repeated 2-3 times for each HIWATE organic extract.

5.2.9. Statistical Analyses

For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to determine if the HIWATE sample induced a statistically significant level of cell death at a specific concentration factor. If a significant F value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was performed to identify the lowest cytotoxic concentration factor. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha = 0.05$.

For the SCGE assay, the %Tail DNA values are not normally distributed, which limits the use of parametric statistics [38]. The mean %Tail DNA value for each microgel was calculated and these values were averaged among all of the microgels for each HIWATE sample concentration factor. Averaged mean values express a normal distribution according to the central limit theorem [38]. A one-way ANOVA test was conducted on these averaged %Tail DNA values [39]. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power ≥ 0.8 at $\alpha = 0.05$.

The mammalian cell cytotoxicity and genotoxicity analyses were compared with the following analytical chemical metrics: (i) the numbers of DBPs identified in each HIWATE sample, (ii) the chromatographic peak area for the entire sample, (iii) peak areas for specific classes of

DBPs, (iv) the total concentration of 21 selected DBPs and, (v) concentrations of specific DBP classes within the group of 21 DBPs. A Pearson's Product Moment correlation test was conducted.

5.3. RESULTS AND DISCUSSION

5.3.1. Chemical Analyses

Over 90 DBPs were identified in the samples, including several haloacids (including 3- and 4-carbon acids and di-acids), halophenols, haloamides, halonitromethanes, haloketones, haloaldehydes, and haloalkenes (Table 5.2). Approximately 300 chromatographic peaks were observed in the original GC/MS chromatograms including DBPs and other compounds present in the raw waters prior to disinfection (Figure 5.2). With GC X GC-TOF-MS analyses, these peaks were resolved into >1000 peaks (Figure 5.3). Several DBPs identified were not in mass spectral library databases and these identifications were made through the methods outlined previously utilizing Massworks software. Several new DBPs were presumptively identified, including cis- and trans-2,3-dibromo-3-chloropropenoic acid, 3,3-dibromo-2-chloropropenoic acid, and several halophenols and haloalkenes. A total of 21 target DBPs, including 4 U.S-regulated THMs, 9 HAAs, 4 haloacetonitriles (HANs), 2 haloketones (HKs), trichloroacetaldehyde (chloral hydrate), and trichloronitromethane (chloropicrin) were quantified (Table 5.3).

Substantial differences were observed in the DBPs from the different locations. As expected, drinking waters from coastal Spain (Barcelona and Valencia) had relatively high DBP levels with many brominated (and some iodinated) species due to higher levels of total organic

carbon (TOC), bromide and iodide in their source waters (surface water), as well as the use of chlorine as a disinfectant. Drinking waters from coastal Spain averaged 90 and 33 μg/L for THM4 and HAA9, respectively (Table 5.3). In contrast, drinking water from Modena, Italy had fewer DBPs and those present were at much lower levels; these were primarily chlorine-containing species. The source water for Modena is a low-TOC groundwater that is treated with low chlorine dioxide doses (0.1 mg/L), which forms fewer DBPs as compared to other disinfectants [4, 40-42]. None of the 21 target DBPs were detected in the drinking water from Modena, but a few were detected in the broad screen analyses due to lower detection limits. Drinking water from other locations (samples 4, 6, 8-11, Table 5.1) expressed intermediate DBP levels with a mix of chloro-bromo species probably due to lower levels of bromide and TOC in their source waters as compared to waters from costal Spain (Table 5.2 and Table 5.3).

Of the N-DBPs [43], haloacetonitriles and haloamides were prevalent in drinking waters from coastal cities in Spain (samples 1-3, 7), which involved treatment with chlorine, alone or in combination with ozone or chlorine dioxide. Previous research demonstrated that ozonation increased the formation of halonitromethanes when used prior to chlorination or chloramination [44-46]. While chloramination increases the formation of some N-DBPs [6], none of the cities in this study employed chloramines.

5.3.2. CHO Cell Chronic Cytotoxicity

CHO cell chronic cytotoxicity analyses of each HIWATE sample are summarized in Table 5.4. The concentration factor is the fold concentration of the isolated organic material as compared to the original water. The lowest concentration factor of each sample which induced

a statistically significant reduction in cell density as compared to its concurrent negative control was determined by an ANOVA test statistic. The data from replicated experiments were averaged and plotted (Figure 5.4, Figures 5.5-5.15); regression analyses were used to calculate the LC_{50} (%C½) value for each sample. Based on the LC_{50} values, the descending rank order of chronic cytotoxicity was, sample 3 > sample 1 > sample 2 ≈ sample 4 > sample 7 > sample 10 > sample 9 > sample 8 ≈ sample 11 > sample 6 > sample 5. Samples from Barcelona, Spain were ranked as the 3 most cytotoxic. We calculated the cytotoxicity index value ($LC_{50}^{-1} \times 1000$) for each HIWATE sample (Figure 5.16, Table 5.5).

5.3.3. CHO Cell Acute Genotoxicity

CHO cell acute genotoxicity analyses of each HIWATE sample are summarized in Table 4.6. The lowest genotoxic concentration factor was that which induced a statistically significant amount of genomic DNA damage as compared to the concurrent negative control. Figure 5.17 (individual plots are shown in Figures 5.18-5.28) illustrates the concentration-response curves for the HIWATE samples. Based on 50% Tail DNA values, the descending rank order of genotoxicity was, sample 10 > sample 4 > sample 7 > sample 1 ≈ sample 2 > sample 3 > sample 9 > sample 11 > sample 6 > sample 8 >> sample 5. We calculated the genotoxic index value as 50% Tail $DNA^{-1} \times 10^4$ for each sample (Figure 5.29, Table 5.5).

5.3.4. Correlation of Toxicology, Chemistry and Epidemiology

To investigate correlations between DBP occurrence and DBP classes with mammalian cell toxicity, we applied a Pearson's Product Moment statistical test [38]. The cytotoxic potency index values statistically significantly correlated with the number of identified DBPs (r = 0.78; P

≤ 0.005, Table 5.7) and the level of 21 target DBPs (r = 0.77; $P \leq 0.006$, Table 4.3). The genotoxic potency index values were not correlated with either of these metrics or with any DBP chemical class (Table 5.3 and Table 5.7). Interestingly, the cytotoxicity and genotoxicity indices indicated a good correlation (r = 0.74; $P \leq 0.009$). The cytotoxic potency index showed a good correlation with the U.S-regulated DBPs (r = 0.78; $P \leq 0.006$) and unregulated DBPs (r = 0.60; $P \leq 0.05$; Table 4.3).

Cytotoxicity was significantly correlated with the relative concentrations of the following DBP classes: THMs (r = 0.74; $P \leq 0.01$), haloacids (r = 0.75; $P \leq 0.008$), other monoacids (r = 0.68; $P \leq 0.021$), halodiacids (r = 0.80; $P \leq 0.003$), haloamides (r = 0.68; $P \leq 0.021$), haloaromatics (r = 0.64; $P \leq 0.035$), brominated (r = 0.68; $P \leq 0.022$), chlorinated (r = 0.78; $P \leq 0.005$) , and iodinated (r = 0.82; $P \leq 0.002$) DBPs (Table 5.8). There were no statistically significant correlations with genotoxicity and the above DBP classes, although there were associations or trends in relationships between genotoxicity and the relative concentrations of haloacids (r = 0.54; $P \leq 0.088$), haloaromatics (r = 0.52; $P \leq 0.103$), chlorinated (r = 0.56; $P \leq 0.073$) and iodinated (r = 0.53; $P \leq 0.093$) DBPs (Table 5.8). It should be noted that some highly polar components might have been missed by GC/MS and this may explain, in part, the reduced correlation seen with the genotoxicity data and analytical chemistry of the water samples. Recently several papers have been published on novel methods to detect polar iodinated/brominated DBPs [47-49].

Epidemiology results from Lithuania were recently published [50] and a dose-response relationship for the THM internal dose and risk for LBW was reported. The authors also found

slight an increase in the risk of SGA related to elevated internal doses of THMs during pregnancy. We expanded comparisons to include the number of identified DBPs and the toxic potency indices from the current study with these epidemiologic measurements. We compared prevalence of LBW and SGA for the high THM exposure group which corresponded to HIWATE sample 4 (this study) and a low THM exposure group which corresponded to sample 6 (this study). We found that the prevalence of LBW, SGA, the number of identified DBPs the level of DBPs, the cytotoxic potency, and the genotoxic potency were all higher for sample 4 versus sample 6 (Figure 5.16 and Figure 5.29, Table 5.4, Tables 5.6-5.7, and Table 5.9). This relationship suggests a coherence association between the analytical chemistry, the *in vitro* toxicology, and the epidemiologic results and support epidemiologic findings for an association between adverse reproductive effects and exposure to DBPs.

Epidemiology results in Spain were recently published that included 3 sites of the HIWATE project corresponding to samples 3, 7 and 9 of this study [51]. The authors focused on maternal exposure to THMs and its association with birth weight, SGA, LBW, and preterm delivery. Although the authors found that residential THM exposure during pregnancy driven by inhalation and dermal contact routes was not associated with birth weight, SGA, LBW, or preterm delivery in Spain, we expanded comparisons to include the number of identified DBPs and the toxic potency indices from the current study with these epidemiologic measurements. There was a trend with higher SGA levels and higher numbers of identified DBPs, and higher genotoxicity and cytotoxicity index values (Table 5.9). An epidemiological study from Rennes, France (HIWATE) concluded that THM and haloacetic acid biomarkers of disinfection by-product exposure suggested prenatal exposure affects fetal growth, but the causal agent or agents

remain to be identified [52]. From these studies it appears that expanding the chemical and toxicological characterization of water samples may enhance the resolving power of epidemiological investigations. It should be noted that the drinking water samples for the epidemiologic analyses and the current chemical/toxicological evaluations were not collected at the same time.

5.4. CONCLUSION

This was the first study that integrated quantitative *in vitro* toxicological data with analytical chemistry and human epidemiologic data for drinking water DBPs. This project focused on the relationship of the occurrence and concentration of DBPs with mammalian cell toxicity. The range of the number of DBPs identified and their levels reflect the diverse collection sites, different disinfection processes, and the different characteristics of the source waters. CHO cytotoxicity was well correlated with the numbers of DBPs identified and the levels of DBP chemical classes. Although there was a clear difference in genotoxic responses, these data did not correlate well with chemical analyses of the HIWATE samples. Thus, the agents responsible for the genomic DNA damage observed in the HIWATE samples may be due to unresolved associations of combinations of identified DBPs, unknown emerging DBPs that were not identified, or other toxic water contaminants.

We are continuing to compare the epidemiology with the *in vitro* toxicity and analytical chemistry analyses. Future study will investigate the possible association between chronic cytotoxicity, acute genotoxicity, multivariate comparisons of identified DBPs and epidemiology

across the entire HIWATE program. We plan to compare other *in vitro* and molecular toxicity metrics and rates of adverse pregnancy measurements. Finally, we propose to determine the contribution of source water, and disinfection chemistry to the observed toxicity and epidemiology results and develop solutions to protect the public health and the environment.

References

1. Cutler, D. a. M., G. , The Role of Public Health Improvements in Health Advances: The Twentieth-Century United States. In *Demography*, 2005; Vol. 42, pp 1-22.
2. Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Res.* **2007,** *41*, (8), 1667-1678.
3. Reckhow, D. A.; Linden, K. G.; Kim, J.; Shemer, H.; Makdissy, G., Effect of UV treatment on DBP formation. *J. Am.Water Works Assoc.* **2010,** *102*, (6), 100-113.
4. Zhang, X.; Echigo, S.; Minear, R. A.; Plewa, M. J., Characterization and comparison of disinfection by-products of four major disinfectants. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 299-314.
5. Richardson, S. D., Disinfection by-products: formation and occurrence in drinking water. In *Encyclopedia of Environmental Health*, Nriagu, J. O., Ed. Elsevier: Burlington, 2011; Vol. 1, pp 110-136.
6. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; Demarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutat. Res.* **2007,** *636*, (1-3), 178-242.
7. Krasner, S. W., The formation and control of emerging disinfection by-products of health concern. *Philos. Transact. A Math. Phys. Eng. Sci.* **2009,** *367*, (1904), 4077-4095.
8. Ueno, H.; Moto, T.; Sayato, Y.; Nakamuro, K., Disinfection by-products in the chlorination of organic nitrogen compounds: by-products from kynurenine. *Chemosphere* **1996,** *33*, (8), 1425-1433.
9. Ye, B.; Wang, W.; Yang, L.; Wei, J.; E, X., Formation and modeling of disinfection by-products in drinking water of six cities in China. *J. Environ. Monit.* **2011,** *13*, (5), 1271-1275.
10. U. S. Environmental Protection Agency, National primary drinking water regulations: Stage 2 disinfectants and disinfection byproducts rule. *Fed. Reg.* **2006,** *71*, (2), 387-493.
11. Bull, R. J.; Reckhow, D. A.; Li, X.; Humpage, A. R.; Joll, C.; Hrudey, S. E., Potential carcinogenic hazards of non-regulated disinfection by-products: haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, halonitriles, and heterocyclic amines. *Toxicology* **2011,** *286*, (1-3), 1-19.
12. Costet, N.; Villanueva, C. M.; Jaakkola, J. J.; Kogevinas, M.; Cantor, K. P.; King, W. D.; Lynch, C. F.; Nieuwenhuijsen, M. J.; Cordier, S., Water disinfection by-products and bladder cancer: is there a European specificity? A pooled and meta-analysis of European case-control studies. *Occup. Environ. Med.* **2011,** *68*, (5), 379-385.
13. Rahman, M. B.; Driscoll, T.; Cowie, C.; Armstrong, B. K., Disinfection by-products in drinking water and colorectal cancer: a meta-analysis. *Int. J. Epidemiol.* **2010,** *39*, (3), 733-745.
14. Villanueva, C. M.; Cantor, K. P.; Cordier, S.; Jaakkola, J. J.; King, W. D.; Lynch, C. F.; Porru, S.; Kogevinas, M., Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* **2004,** *15*, (3), 357-367.

15. Villanueva, C. M.; Cantor, K. P.; Grimalt, J. O.; Malats, N.; Silverman, D.; Tardon, A.; Garcia-Closas, R.; Serra, C.; Carrato, A.; Castano-Vinyals, G.; Marcos, R.; Rothman, N.; Real, F. X.; Dosemeci, M.; Kogevinas, M., Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am. J. Epidemiol.* **2007,** *165*, (2), 148-156.
16. Grellier, J.; Bennett, J.; Patelarou, E.; Smith, R. B.; Toledano, M. B.; Rushton, L.; Briggs, D. J.; Nieuwenhuijsen, M. J., Exposure to disinfection by-products, fetal growth, and prematurity: a systematic review and meta-analysis. *Epidemiology* **2010,** *21*, (3), 300-313.
17. Hinckley, A. F.; Bachand, A. M.; Reif, J. S., Late pregnancy exposures to disinfection by-products and growth-related birth outcomes. *Environ. Health Perspect.* **2005,** *113*, (12), 1808-1813.
18. Wright, J. M.; Schwartz, J.; Dockery, D. W., Effect of trihalomethane exposure on fetal development. *Occup. Environ. Med.* **2003,** *60*, (3), 173-180.
19. Yang, C. Y.; Xiao, Z. P.; Ho, S. C.; Wu, T. N.; Tsai, S. S., Association between trihalomethane concentrations in drinking water and adverse pregnancy outcome in Taiwan. *Environ. Res.* **2007,** *104*, (3), 390-395.
20. Hunter, E. S.; Rogers, E. H.; Schmid, J. E.; Richard, A., Comparative effects of haloacetic acids in whole embryo culture. *Teratology* **1996,** *54*, (2), 57-64.
21. Narotsky, M. G.; Best, D. S.; McDonald, A.; Godin, E. A.; Hunter, E. S., 3rd; Simmons, J. E., Pregnancy loss and eye malformations in offspring of F344 rats following gestational exposure to mixtures of regulated trihalomethanes and haloacetic acids. *Reprod. Toxicol.* **2011,** *31*, (1), 59-65.
22. Nieuwenhuijsen, M. J.; Smith, R.; Golfinopoulos, S.; Best, N.; Bennett, J.; Aggazzotti, G.; Righi, E.; Fantuzzi, G.; Bucchini, L.; Cordier, S.; Villanueva, C. M.; Moreno, V.; La Vecchia, C.; Bosetti, C.; Vartiainen, T.; Rautiu, R.; Toledano, M.; Iszatt, N.; Grazuleviciene, R.; Kogevinas, M., Health impacts of long-term exposure to disinfection by-products in drinking water in Europe: HIWATE. *J. Water Health* **2009,** *7*, (2), 185-207.
23. Richardson, S. D., XAD resin extraction of disinfectant by-products drinking water: SOP - RSB-003.1- Revision No. 1. In Environmental Protection Agency: Athens, GA, 2011.
24. Pressman, J. G.; Richardson, S. D.; Speth, T. F.; Miltner, R. J.; Narotsky, M. G.; Hunter, E. S., 3rd; Rice, G. E.; Teuschler, L. K.; McDonald, A.; Parvez, S.; Krasner, S. W.; Weinberg, H. S.; McKague, A. B.; Parrett, C. J.; Bodin, N.; Chinn, R.; Lee, C. F.; Simmons, J. E., Concentration, chlorination, and chemical analysis of drinking water for disinfection byproduct mixtures health effects research: U.S. EPA's Four Lab Study. *Environ. Sci. Technol.* **2010,** *44*, (19), 7184-7192.
25. Chen, P. H.; Richardson, S. D.; Krasner, S. W.; Majetich, G.; Glish, G. L., Hydrogen abstraction and decomposition of bromopicrin and other trihalogenated disinfection byproducts by GC/MS. *Environ. Sci. Technol.* **2002,** *36*, (15), 3362-3371.
26. Wang, Y.; Liao, X. H.; Gu, Y.; Chen, R.; Zeng, J., [Preliminary study on oxygen content monitoring for port wine stains during PDT using diffuse reflection spectra]. *Guang Pu Xue Yu Guang Pu Fen Xi* **2010,** *30*, (12), 3363-3366.

27. EPA Method 551.1, Determination of chlorination disinfection byproducts, chlorinated solvents, and halogenated pesticides/herbicides in drinking water by liquid-liquid extraction and gas chromatography with electron-capture detection, Revision 1.0. In National Exposure Research Laboratory, USEPA Office of Research and Development.: Cincinnati, OH., 1995.
28. EPA Method 552.3, Determination of haloacetic acids and dalapon in drinking water by liquid-liquid microextraction, derivatization and gas chromatography with electron capture detection, Revision 1.0. In U.S. EPA Office of Reserach and Development, Cincinnati OH: 2003.
29. Hsie, A. W.; Brimer, P. A.; Mitchell, T. J.; Gosslee, D. G., The dose-response relationship for ultraviolet-light-induced mutations at the hypoxanthine-guanine phosphoribosyltransferase locus in Chinese hamster ovary cells. *Somatic Cell Genet.* **1975,** *1*, (4), 383-389.
30. Tindall, K. R.; Stankowski, L. F., Jr.; Machanoff, R.; Hsie, A. W., Detection of deletion mutations in pSV2gpt-transformed cells. *Mol. Cell Biol.* **1984,** *4*, (7), 1411-1415.
31. Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J., Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998,** *32*, (4), 360-368.
32. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
33. Fairbairn, D. W.; Olive, P. L.; O'Neill, K. L., The comet assay: a comprehensive review. *Mutat. Res.* **1995,** *339*, (1), 37-59.
34. Tice, R. R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J. C.; Sasaki, Y. F., Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **2000,** *35*, (3), 206-221.
35. Rundell, M. S.; Wagner, E. D.; Plewa, M. J., The comet assay: genotoxic damage or nuclear fragmentation? *Environ. Mol. Mutagen.* **2003,** *42*, (2), 61-67.
36. Wagner, E. D.; Plewa, M. J., Microplate-based comet assay. In *The Comet Assay in Toxicology*, Dhawan, A.; Anderson, D., Eds. Royal Society of Chemistry: London, 2009; pp 79-97.
37. Phillips, H. J., Dye exclusion tests for cell viability. In *Tissue Culture: Methods and Applications*, Kruse, P. F.; Patterson, M. J., Eds. Academic Press: New York, 1973; p 406.
38. Box, G. E. P.; Hunter, W. G.; Hunter, J. S., *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*. Wiley & Sons Inc.: New York, NY., 1978.
39. Lovell, D. P.; Omori, T., Statistical issues in the use of the comet assay. *Mutagenesis* **2008,** *23*, (3), 171-182.
40. Richardson, S. D.; Rav-Acha, C.; Simpson, G. C., Chlorine dioxide chemistry, reactions, and disinfection by-products. In *State of the Science of Chlorine Dioxide in Drinking Water;*, Gates, D.; Ziglio, G.; Ozekin, K., Eds. Water Research Foundation and Fondazione AMGA: Denver, CO., 2009; pp 21-49.
41. Richardson, S. D.; Thruston, A. D., Jr.; Rav-Acha, C.; Groisman, L.; Popilevsky, I.; Juraev, O.; Glezer, V.; McKague, A. B.; Plewa, M. J.; Wagner, E. D., Tribromopyrrole, brominated acids, and other disinfection byproducts produced by disinfection of drinking water rich in bromide. *Environ. Sci. Technol.* **2003,** *37*, (17), 3782-3793.

42. Weinberg, H. S.; Krasner, S. W.; Richardson, S. D.; Thruston, A. D., Jr. *The Occurrence of Disinfection By-Products (DBPs) of Health Concern in Drinking Water: Results of a Nationwide DBP Occurrence Study*; EPA/600/R02/068; U.S. Environmental Protection Agency National Exposure Research Laboratory: Athens, GA., 2002; pp 1-460.
43. Plewa, M. J.; Wagner, E. D.; Muellner, M. G.; Hsu, K. M.; Richardson, S. D., Comparative mammalian cell toxicity of N-DBPs and C-DBPs. In *Occurrence, formation, health effects and control of disinfection by-products in drinking water*, Karanfil, T.; Krasner, S. W.; Westerhoff, P.; Xie, Y., Eds. American Chemical Society: Washington, D.C., 2008; Vol. 995, pp 36-50.
44. Choi, J. H.; Pfeifer, G. P., DNA damage and mutations produced by chloroacetaldehyde in a CpG-methylated target gene. *Mutat. Res.* **2004,** *568*, (2), 245-256.
45. Hu, J.; Song, H.; Addison, J. W.; Karanfil, T., Halonitromethane formation potentials in drinking waters. *Water Res.* **2010,** *44*, (1), 105-114.
46. Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D., Jr., The occurrence of a new generation of disinfection by-products. *Environ. Sci. Technol.* **2006,** *40*, (23), 7175-7185.
47. Ding, G.; Zhang, X., A picture of polar iodinated disinfection byproducts in drinking water by (UPLC/)ESI-tqMS. *Environ. Sci. Technol.* **2009,** *43*, (24), 9287-9293.
48. Zhai, H.; Zhang, X., Formation and decomposition of new and unknown polar brominated disinfection byproducts during chlorination. *Environ. Sci. Technol.* **2011,** *45*, (6), 2194-2201.
49. Zhang, X.; Talley, J. W.; Boggess, B.; Ding, G.; Birdsell, D., Fast selective detection of polar brominated disinfection byproducts in drinking water using precursor ion scans. *Environ. Sci. Technol.* **2008,** *42*, (17), 6598-6603.
50. Grazuleviciene, R.; Nieuwenhuijsen, M. J.; Vencloviene, J.; Kostopoulou-Karadanelli, M.; Krasner, S. W.; Danileviciute, A.; Balcius, G.; Kapustinskiene, V., Individual exposures to drinking water trihalomethanes, low birth weight and small for gestational age risk: a prospective Kaunas cohort study. *Environ. Health* **2011,** *10*, 32.
51. Villanueva, C. M.; Gracia-Lavedan, E.; Ibarluzea, J.; Santa Marina, L.; Ballester, F.; Llop, S.; Tardon, A.; Fernandez, M. F.; Freire, C.; Goni, F.; Basagana, X.; Kogevinas, M.; Grimalt, J. O.; Sunyer, J.; Project, I., Exposure to trihalomethanes through different water uses and birth weight, small for gestational age, and preterm delivery in Spain. *Environ. Health Perspect.* **2011,** *119*, (12), 1824-1830.
52. Costet, N.; Garlantezec, R.; Monfort, C.; Rouget, F.; Gagniere, B.; Chevrier, C.; Cordier, S., Environmental and urinary markers of prenatal exposure to drinking water disinfection by-products, fetal growth, and duration of gestation in the PELAGIE birth cohort (Brittany, France, 2002-2006). *Am. J. Epidemiol.* **2012,** *175*, (4), 263-275.

TABLES AND FIGURES

Table 5.1. HIWATE water sampling locations and applied disinfection methods.

Sample Number	Sampling Location (Site)	Disinfection Method
HIWATE 1	Barcelona, Spain (Badalona)	Cl_2-Cl_2
HIWATE 2	Barcelona, Spain (Hospitalet del Llobregat)	Blend of Cl_2-Cl_2 , Cl_2-O_3-Cl_2, Desal-RO-ClO_2
HIWATE 3	Barcelona, Spain (Sabadell)	Blend of (ClO_2/Cl_2)-Cl_2, Cl_2-Cl_2
HIWATE 4	Kaunas, Lithuania (Petruniusai)	Cl_2
HIWATE 5	Modena, Italy	ClO_2
HIWATE 6	Kaunas, Lithuania (Viciunai)	Cl_2
HIWATE 7	Valencia, Spain	Cl_2-Cl_2
HIWATE 8	Rennes, France	O_3-Cl_2
HIWATE 9	Asturias, Spain	Cl_2
HIWATE 10	Bradford, U.K. (Shipley)	Cl_2
HIWATE 11	Bradford, U.K. (Airedale)	Cl_2

Cl_2 = chlorination, O_3 = ozonation, ClO_2 = chlorine dioxide, Desal-RO = desalination with reverse osmosis.

Table 5.2. DBPs identified from the broadscreen anlaysis[a].

	HIWATE Sample Number and Relative Concentrations of DBPs										
Regulated THMs[b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Bromodichloromethane	71957	43619	42668	17579	ND	ND	50493	36343	48465	35449	26330
Dibromochloromethane	99952	103640	118499	7429	1154	1568	123682	71944	43365	14724	22480
Bromoform	134734	150331	158731	200	1000	500	107089	61451	17688	500	2371
Unregulated halomethanes											
Dichloroiodomethane	3885	1768	662	8578	ND	780	3176	3133	ND	ND	2917
Bromochloroiodomethane	2990	5774	3651	2220	ND	ND	5654	2927	5568	ND	484
Dibromoiodomethane	7548	7829	12034	172	ND	ND	4421	2348	1440	ND	ND
Haloalkenes											
Hexachlorocyclopentadiene	100	ND	100	4236	ND	ND	100	ND	6921	3777	1363
Bromopentachlorocyclopentadiene	ND	ND	50	400	ND	ND	50	50	500	200	100
Dibromotetrachlorocyclopentadiene	ND	ND	ND	1308	ND	ND	200	ND	200	300	300

Table 5.2. (Continued)

Haloacetic acids											
Bromoacetic acid	2834	3405	4402	ND	ND	ND	3097	1321	ND	ND	ND
Dichloroacetic acid	27347	8358	19066	24875	437	471	32384	22568	40491	61010	5000
Bromochloroacetic acid	23808	12111	24172	3840	ND	385	44492	27264	ND	3299	ND
Dibromoacetic acid	106492	99125	109402	ND	ND	ND	128964	73451	ND	ND	ND
Trichloroacetic acid	46087	21075	44130	25729	436	788	25146	13146	110081	72121	38623
Bromodichloroacetic acid	24640	16993	27003	2885	ND	ND	45921	18504	21768	4521	6681
Dibromochloroacetic acid	74411	106576	110691	ND	ND	ND	100516	32733	ND	ND	ND
Other Mono-haloacids											
2-Bromopropanoic acid	2440	1658	4969	ND	ND	ND	3428	1359	ND	ND	ND
2,2-Dichloropropanoic acid	6229	1733	6785	1975	ND	ND	5213	2066	5760	13578	5641
2,3-Dibromopropanoic acid	26425	17717	21135	ND	ND	ND	9638	2511	ND	ND	ND
3,3-Dichloropropenoic acid	756	2066	2033	ND	ND	ND	1582	746	100	570	1401
3,3-Bromochloropropenoic acid	ND	2368	1744	ND	ND	ND	1717	610	ND	ND	279

Table 5.2. (Continued)

Other Mono-haloacids (continued)											
trans-2,3-Bromochloropropenoic acid	ND	3152	1582	ND	ND	ND	2661	1124	ND	ND	100
cis-2,3-Dibromopropenoic acid	100	63129	62735	ND	ND	ND	25869	4691	ND	ND	ND
trans-2,3-Dibromopropenoic acid	100	6484	3875	ND	ND	ND	650	ND	ND	ND	ND
2-Bromo-3,3-dichloropropenoic acid	100	3780	3367	ND	ND	ND	3220	2517	2093	2884	2684
(E)-3-Bromo-2,3-dichloropropenoic acid	100	5238	2640	ND	ND	ND	1869	1466	1497	2041	1804
(Z)-3-Bromo-2,3-dichloropropenoic acid	100	3353	1694	ND	ND	ND	1678	771	1500	5723	2999
3,3-Dibromo-2-chloropropenoic acid	200	8750	7783	ND	ND	ND	6058	4197	ND	ND	ND
(E)-2,3-Dibromo-3-chloropropenoic acid	200	8750	7783	ND	ND	ND	7000	1631	ND	ND	ND
(Z)-2,3-Dibromo-3-chloropropenoic acid	200	8750	7783	ND	ND	ND	11257	6061	ND	ND	100
Tribromopropenoic acid	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
cis-2-Bromobutenoic acid	4763	2948	6296	ND	ND	ND	6499	ND	ND	ND	ND
trans-2-Bromobutenoic acid	3621	16340	15316	ND	ND	ND	1044	ND	ND	ND	ND
trans-2,3-Dibromobutenoic acid	200	14950	2711	ND	ND	ND	1805	278	ND	ND	ND
Dibromohexanoic acid	1000	1500	1300	ND	ND	ND	200	100	ND	ND	ND

Table 5.2. (Continued)

Other Mono-haloacids (continued)											
Bromoheptanoic acid, isomer #1	7817	3227	8193	ND	ND	ND	11428	2247	ND	749	ND
Bromoheptanoic acid, isomer #2	2000	1500	3569	ND	ND	ND	6386	1511	ND	ND	ND
Bromooctanoic acid	3070	3316	9408	ND	ND	ND	12749	2037	ND	781	50
Halo-di-acids											
cis-2,3-Dichlorobutenedioic acid	100	100	4361	2283	ND	ND	3213	1806	1740	572	236
trans-2,3-Dichlorobutenedioic acid	100	ND	6005	3500	ND	ND	7228	3031	5404	4773	4109
trans-2,3-Dibromobutenedioic acid	24438	21022	38116	ND	ND	ND	17935	3902	5531	ND	ND
Halonitriles											
Dichloroacetonitrile	22941	8062	5885	15406	ND	ND	9632	5199	38435	24513	4016
Bromochloroacetonitrile	19192	18931	19149	2969	ND	ND	30593	19087	10733	4023	769
Dibromoacetonitrile	84107	48881	51157	ND	ND	ND	156558	28079	4045	ND	ND
Dibromochloroacetonitrile	9092	3409	3196	ND	ND	ND	3816	3046	731	ND	ND

Table 5.2. (Continued)

Halonitriles (continued)											
Tribromoacetonitrile	10005	8517	7848	ND	ND	ND	10169	3566	ND	ND	ND
2,2-Dibromopropanenitrile	1941	1983	1995	ND	ND	ND	2474	400	ND	ND	ND
Haloketones											
1,1-Dichloropropanone	ND	ND	ND	3098	ND	ND	ND	ND	ND	ND	ND
1-Bromo-1-chloropropanone	1439	2331	854	965	ND	ND	1702	4180	ND	ND	ND
1,1-Dibromopropanone	16332	18195	ND	ND	ND	ND	2170	6951	ND	ND	ND
1,1,1-Trichloropropanone	11,589	4046	5068	8908	394	ND	11370	10932	24592	13469	683
1-Bromo-1,1-dichloropropanone	7886	3054	967	1262	ND	ND	5595	7068	ND	ND	ND
1,1,1-Tribromopropanone	7500	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,1,3-Tribromopropanone	1546	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,1,1,3-Tetrachloropropanone	2688	ND	1655	1095	ND	ND	ND	ND	16254	1864	1354
1,1,3,3-Tetrachloropropanone	ND	ND	ND	2000	ND	ND	ND	ND	24351	3356	200
1,1,3,3-Tetrabromopropanone	5317	914	1877	ND	ND	ND	200	ND	ND	ND	ND

Table 5.2. (Continued)

Haloketones (continued)											
Pentachloropropanone	9277	ND	3125	3338	ND	ND	2000	ND	46254	8919	961
3-Bromo-2-butanone	16880	960	388	ND	ND	ND	200	946	ND	ND	ND
Haloaldehydes											
Bromodichloroacetaldehyde	3240	7354	526	ND	ND	ND	3365	2102	1215	500	ND
Dibromochloroacetaldehyde	16301	21083	11149	ND	ND	ND	3438	3545	593	ND	ND
Tribromoacetaldehyde	28471	22575	11791	ND	ND	ND	3318	1867	ND	ND	ND
Halonitromethanes											
Dichloronitromethane	ND	ND	ND	ND	ND	ND	ND	ND	ND	8390	ND
Bromochloronitromethane	ND	3602	1092	ND	ND	ND	ND	2049	ND	ND	ND
Dibromonitromethane	11004	18400	6702	ND	ND	ND	3780	5832	ND	ND	ND
Trichloronitromethane (Chloropicrin)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1000
Bromodichloronitromethane	ND	ND	ND	ND	ND	ND	ND	1693	1169	983	ND

Table 5.2. (Continued)

Halonitromethanes (continued)											
Dibromochloronitromethane	ND	ND	ND	ND	ND	ND	ND	9999	400	ND	ND
Tribromonitromethane	12857	683	4422	ND	ND	ND	18563	13283	ND	ND	ND
Haloamides											
Dichloroacetamide	17151	ND	12794	3000	ND	ND	200	7147	ND	ND	ND
Bromochloroacetamide	16218	10291	10254	416	ND	ND	ND	3592	ND	ND	ND
Dibromoacetamide	109188	94776	105496	ND	ND	ND	106659	61437	ND	ND	ND
Trichloroacetamide	11204	2162	7720	1150	ND	ND	9459	4165	5909	4259	ND
Bromodichloroacetamide	62406	144668	124452	ND	ND	ND	83074	5000	ND	ND	ND
Dibromochloroacetamide	7317	13211	11800	ND	ND	ND	27653	8228	ND	ND	ND
Tribromoacetamide	13435	7442	12383	ND	ND	ND	19549	3811	ND	ND	ND
Haloaromatics											
Dichlorobenzene	2000	4944	5223	1633	2563	1325	3370	2038	2220	200	382

Table 5.2. (Continued)

Haloaromatics (continued)											
Dibromophenol, isomer #1	4680	3427	2550	ND	ND	ND	5934	3548	2500	500	500
Dibromophenol, isomer #2	2368	277	4714	2707	ND	1224	4820	1346	ND	ND	ND
Trichlorophenol, isomer #1	1958	2591	2720	1173	879	ND	6819	2000	500	200	4498
Trichlorophenol, isomer #2	4263	3558	3055	3350	1039	782	8811	4300	ND	ND	ND
Trichlorophenol, isomer #3	3767	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bromochlorophenol, isomer #1	40405	91241	40609	17142	10239	9279	51408	7003	19147	ND	ND
Bromochlorophenol, isomer #2	66243	67840	68444	42865	ND	42595	57520	8109	41944	500	300
Bromochlorophenol, isomer #3	ND	6432	2843	2000	ND	2000	8948	1500	ND	ND	ND
Bromodichlorophenol, isomer #1	100	ND	ND	ND	100	100	1146	100	500	200	6393
Bromodichlorophenol, isomer #2	100	100	100	1575	492	680	8306	100	300	100	ND
Bromodichlorophenol, isomer #3	100	ND	100	1025	ND	100	2076	ND	ND	ND	2448
Dibromochlorophenol, isomer #1	1980	775	2477	ND	ND	ND	1958	2694	2541	200	ND
Dibromochlorophenol, isomer #2	1573	1581	2155	4921	ND	200	8786	5445	10651	200	910
Dibromochlorophenol, isomer #3	1361	ND	1324	2776	ND	200	1516	5450	3343	200	1105

Table 5.2. (Continued)

Haloaromatics (continued)											
Tribromophenol, isomer #1	933	500	1291	ND	100	ND	500	500	ND	ND	ND
Tribromophenol, isomer #2	7179	4287	5852	500	200	100	4787	3201	3873	500	2580
Tribromophenol, isomer #3	100	100	100	ND	ND	ND	ND	ND	ND	ND	ND
Dibromoimidazole	642	ND	921	ND	ND	ND	ND	ND	ND	ND	ND

[a] Numbers represent measured GC/MS peak areas; ND = not detected; compound names in italics were confirmed through the analysis of authentic standards; all other identifications should be considered tentative. [b] Note: Chloroform was detected in corresponding quantitative analyses of volatile analytes, but was not detected in broadscreen analyses, which are designed for semivolatile analytes.

Table 5.3. Levels of DBPs by chemical classes and correlation with CHO cell cytotoxic potency index and genotoxic potency index.

HIWATE Sample Number	21 DBPs[c] (μg/L)	4 THMs (μg/L)	9 HAAs (μg/L)	4 HANs (μg/L)	2 HKs (μg/L)	CH (μg/L)	CP (μg/L)	U.S.-Regulated DBPs (μg/L)	Unregulated DBPs (μg/L)
1	115	70.9	36.0	6.47	0.21	1.27	<LOD [d]	94.7	20.1
2	91.1	66.8	19.5	4.70	<LOD	<LOD	<LOD	77.1	13.9
3	202	139	51.5	8.88	1.11	1.80	<LOD	168	33.8
4	3.24	3.24	<LOD	<LOD	<LOD	<LOD	<LOD	3.24	<LOD
5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	1.11	1.11	<LOD	<LOD	<LOD	<LOD	<LOD	1.11	<LOD
7	118	83.9	26.7	4.80	<LOD	2.46	<LOD	103	14.6
8	27.8	14.7	13.3	<LOD	<LOD	<LOD	<LOD	26.5	1.25
9	92.9	55.0	<LOD	4.02	6.86	23.2	3.78	55.0	37.9
10	40.6	22.6	13.3	<LOD	3.28	1.43	<LOD	29.0	11.6
11	45.2	29.3	11.6	<LOD	3.08	1.23	<LOD	36.8	8.37
Cytotoxic Potency Index[a]	$r = 0.77$ $P \leq 0.006$	$r = 0.77$ $P \leq 0.006$	$r = 0.75$ $P \leq 0.009$	$r = 0.73$ $P = 0.011$	$r = 0.04$ $P = 0.913$	$r = 0.04$ $P = 0.905$	$r = -0.02$ $P = 0.947$	$r = 0.76$ $P \leq 0.006$	$r = 0.60$ $P = 0.051$
Genotoxic Potency Index[b]	$r = 0.36$ $P = 0.273$	$r = 0.37$ $P = 0.260$	$r = 0.40$ $P = 0.221$	$r = 0.36$ $P = 0.281$	$r = -0.08$ $P = 0.827$	$r = -0.12$ $P = 0.720$	$r = -0.18$ $P = 0.600$	$r = 0.38$ $P = 0.248$	$r = 0.22$ $P = 0.521$

[a] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample. [b] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$. [c] These 21 quantitatively measured DBPs are listed in the text. [d] LOD = limit of detection.

Table 5.4. CHO cell chronic cytotoxicity analyses of the HIWATE samples.

Sample Number	Concentration Factor Range	Lowest Cytotoxic Concentration Factor [a]	LC_{50} Value [b] (Conc. Factor ± SE)	r^2 [c]	ANOVA Test Statistic [d]
HIWATE 1	0 – 150	60	102.7 ± 4.2	0.95	$F_{10, 37} = 58.4$; $P \leq 0.001$
HIWATE 2	0 – 150	70	107.8 ± 3.8	0.97	$F_{10, 37} = 59.2$; $P \leq 0.001$
HIWATE 3	0 – 300	50	79.1 ± 4.1	0.96	$F_{19, 76} = 130$; $P \leq 0.001$
HIWATE 4	0 – 300	22.5	107.5 ± 3.7	0.97	$F_{19, 76} = 134$; $P \leq 0.001$
HIWATE 5	0 – 1000	300	605.8 ± 4.3	0.98	$F_{9, 33} = 69.2$; $P \leq 0.001$
HIWATE 6	0 – 800	300	366.9 ± 4.1	0.99	$F_{10, 37} = 113$; $P \leq 0.001$
HIWATE 7	0 – 350	50	122.1 ± 3.4	0.99	$F_{11, 44} = 212$; $P \leq 0.001$
HIWATE 8	0 – 300	70	162.5 ± 4.8	0.98	$F_{10, 37} = 71.7$; $P \leq 0.001$
HIWATE 9	0 – 300	80	140.0 ± 4.9	0.98	$F_{10, 37} = 90.8$; $P \leq 0.001$
HIWATE 10	0 – 300	80	128.9 ± 4.6	0.97	$F_{11, 40} = 77.9$; $P \leq 0.001$
HIWATE 11	0 – 600	100	164.4 ± 5.2	0.98	$F_{10, 37} = 78.0$; $P \leq 0.001$

[a] Lowest cytotoxic concentration was the lowest concentration factor of the HIWATE sample in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls. [b] The LC_{50} value is the fold concentration factor of the HIWATE sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. The LC_{50} error term was calculated as $\Sigma\bar{x}_{SE}$. [c] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 5.5. Cytotoxicity and genotoxicity index values.

HIWATE Sample Number	Cytotoxicity Index Values $(LC_{50})^{-1}(10^3)$	Genotoxicity Index Values (50% Tail DNA)$^{-1}(10^4)$
HIWATE 1	9.7	8.73
HIWATE 2	9.3	8.71
HIWATE 3	12.6	6.99
HIWATE 4	9.3	9.27
HIWATE 5	1.6	1.77
HIWATE 6	2.7	5.19
HIWATE 7	8.2	8.94
HIWATE 8	6.1	4.53
HIWATE 9	7.1	5.41
HIWATE 10	7.8	9.50
HIWATE 11	6.1	5.26

Table 5.6. CHO cell SCGE genotoxicity analyses of the HIWATE samples.

Sample Number	Concentration Factor Range	Lowest Genotoxic Concentration Factor [a]	50% tail DNA Value [b] (Conc. Factor ±SE)	r^2 [c]	ANOVA Test Statistic [d]
HIWATE 1	0 – 1700	1000	1146 ± 9.1	0.99	$F_{9, 33} = 10.5$; $P \leq 0.001$
HIWATE 2	0 – 2000	1000	1148 ± 3.8	0.99	$F_{10, 37} = 43.6$; $P \leq 0.001$
HIWATE 3	0 – 2000	900	1430 ± 2.0	0.99	$F_{8, 37} = 133$; $P \leq 0.001$
HIWATE 4	0 – 1600	1000	1079 ± 3.3	0.99	$F_{10, 37} = 78.0$; $P \leq 0.001$
HIWATE 5	0 – 3800	3600	5659 ± 1.4	0.74	$F_{10, 38} = 3.14$; $P \leq 0.005$
HIWATE 6	0 – 2200	1600	1925 ± 2.0	0.90	$F_{11, 39} = 20.0$; $P \leq 0.001$
HIWATE 7	0 – 2200	600	1119 ± 2.9	0.95	$F_{9, 26} = 76.3$; $P \leq 0.001$
HIWATE 8	0 – 3000	2000	2206 ± 5.6	0.98	$F_{13, 50} = 9.29$; $P \leq 0.001$
HIWATE 9	0 – 2400	1600	1847 ± 6.9	0.89	$F_{10, 42} = 6.87$; $P \leq 0.001$
HIWATE 10	0 – 1600	400	1052 ± 6.2	0.98	$F_{8, 50} = 7.18$; $P \leq 0.001$
HIWATE 11	0 – 2400	1600	1901 ± 3.7	0.99	$F_{10, 33} = 15.9$; $P \leq 0.001$

[a] The lowest genotoxic concentration was the lowest concentration of the HIWATE sample in the concentration-response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [b] The SCGE 50% Tail DNA value is the HIWATE sample concentration factor determined from a regression analyses of the data that was calculated to induce a 50% SCGE Tail DNA value. The 50% SCGE Tail DNA value error term was calculated as as $\Sigma\bar{x}_{SE}$. [c] r^2 is the coefficient of determination for the regression analysis upon which the SCGE % Tail DNA value was calculated. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 5.7. Description of each HIWATE sample, DBPs identified and gross correlation with the rank order of CHO cell cytotoxicity and genotoxicity.

Sample Number	Number of Identified DBPs	Rank order of Number of Identified DBPs	Rank order of Cytotoxic Potency Index [a]	Rank order of Genotoxic Potency Index [b]
HIWATE 1	86	1	2	4
HIWATE 2	76	5	3	5
HIWATE 3	85	2	1	6
HIWATE 4	41	7	3	2
HIWATE 5	13	11	11	11
HIWATE 6	18	10	10	9
HIWATE 7	83	3	5	3
HIWATE 8	77	4	8	10
HIWATE 9	45	6	7	7
HIWATE 10	41	7	6	1
HIWATE 11	40	9	8	8

Correlation with the rank order of CHO cell cytotoxicity: $r = 0.78$ ($P \leq 0.005$). Correlation with the rank order of CHO cell genotoxicity: $r = 0.52$ ($P = 0.105$). Rank order where 1 is the highest response and 11 is the lowest response. [a] The CHO cell cytotoxic potency index value is in arbitrary units and the value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample. [b] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$ and is presented in arbitrary units.

Table 5.8. Pearson Product Moment correlation analyses of the relative concentrations of each DBP group versus CHO cell chronic cytotoxicity or acute genotoxicity.

Relative Concentration of DBP Class [a]	Cytotoxic Potency Index Value [b] ($LC_{50}^{-1} \times 10^3$)	Genotoxic Potency Index Value [c] (50% tail $DNA^{-1} \times 10^4$)
THMs	$r = 0.74$ $P \leq 0.010$	$r = 0.45$ $P = 0.164$
Haloacids	$r = 0.75$ $P \leq 0.008$	$r = 0.54$ $P = 0.088$
Other monoacids	$r = 0.68$ $P \leq 0.021$	$r = 0.42$ $P = 0.201$
Halodiacids	$r = 0.80$ $P \leq 0.003$	$r = 0.40$ $P = 0.221$
Haloamides	$r = 0.68$ $P \leq 0.021$	$r = 0.45$ $P = 0.170$
Haloaromatics	$r = 0.64$ $P \leq 0.035$	$r = 0.52$ $P = 0.103$
Brominated DBPs	$r = 0.68$ $P \leq 0.022$	$r = 0.46$ $P = 0.154$
Chlorinated DBPs	$r = 0.78$ $P \leq 0.005$	$r = 0.56$ $P = 0.073$
Iodinated DBPs	$r = 0.82$ $P \leq 0.002$	$r = 0.53$ $P = 0.093$

[a] Relative concentration is defined as the integrated area for each chromatographic peak summed for each DBP chemical class. [b] The CHO cell cytotoxic potency index value corresponds to ($LC_{50}^{-1} \times 10^3$) for each HIWATE sample. [c] The CHO cell genotoxic potency index value is the reciprocal HIWATE sample concentration factor that was calculated to induce a 50% SCGE tail DNA value $\times 10^4$.

Table 5.9. General comparisons among published HIWATE epidemiological results and data presented in this study.

Reference	DBP Metric	Epidemiol. Metric	HIWATE Sample No. (this study)	Number of DBPs (this tudy)	CHO Cell Cytotox. Index Value (this study)	CHO Cell Genotox. Index Value (this study)
[50]	Low TTHM [a] (mean = 1.3 µg/L)	3.4% LBW [b] 7.8% SGA [c]	HIWATE 6 Viciunai Lithuania	18	2.7	5.19
[50]	High TTHM [a] (mean = 21.9 µg/L)	5.4% LBW[b] 8.7% SGA[c]	HIWATE 4 Petruniusai Lithuania	41	9.3	9.27
[51]	Sabadell water	11.5 %SGA [c]	HIWATE 3 Sabadell Spain	85	12.6	6.99
[51]	Valencia water	12.2 %SGA [c]	HIWATE 7 Valencia Spain	83	8.2	8.94
[51]	Asturias water	9.0 %SGA [c]	HIWATE 9 Asturias Spain	45	7.1	5.41

[a] TTHM, total THMs; [b]LBW, low birth weight; [c] SGA, small for gestational age [50, 51].

Figure 5.1. HIWATE water sampling locations.

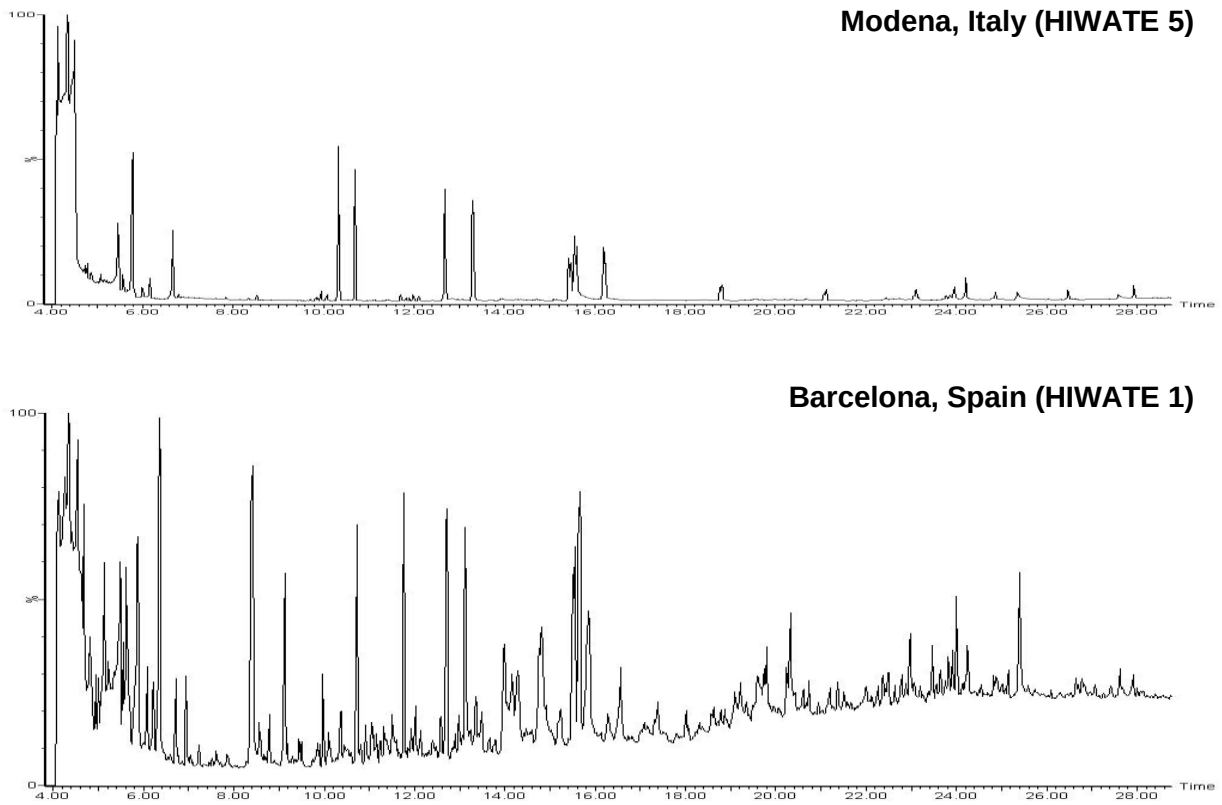


Figure 5.2. Examples of two GC/MS chromatograms.

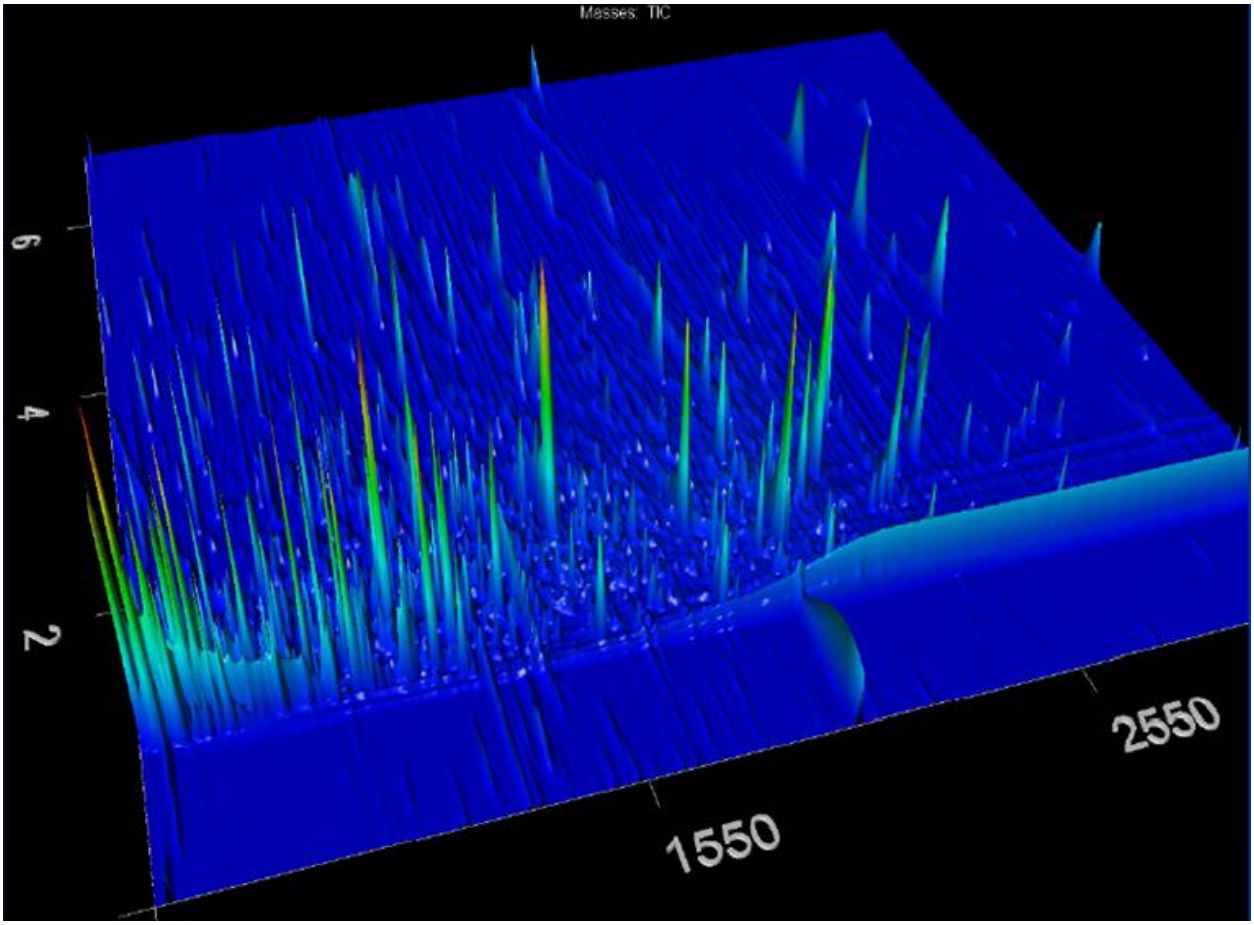


Figure 5.3. Example of a GCxGC-TOF-MS chromatogram (HIWATE 1).

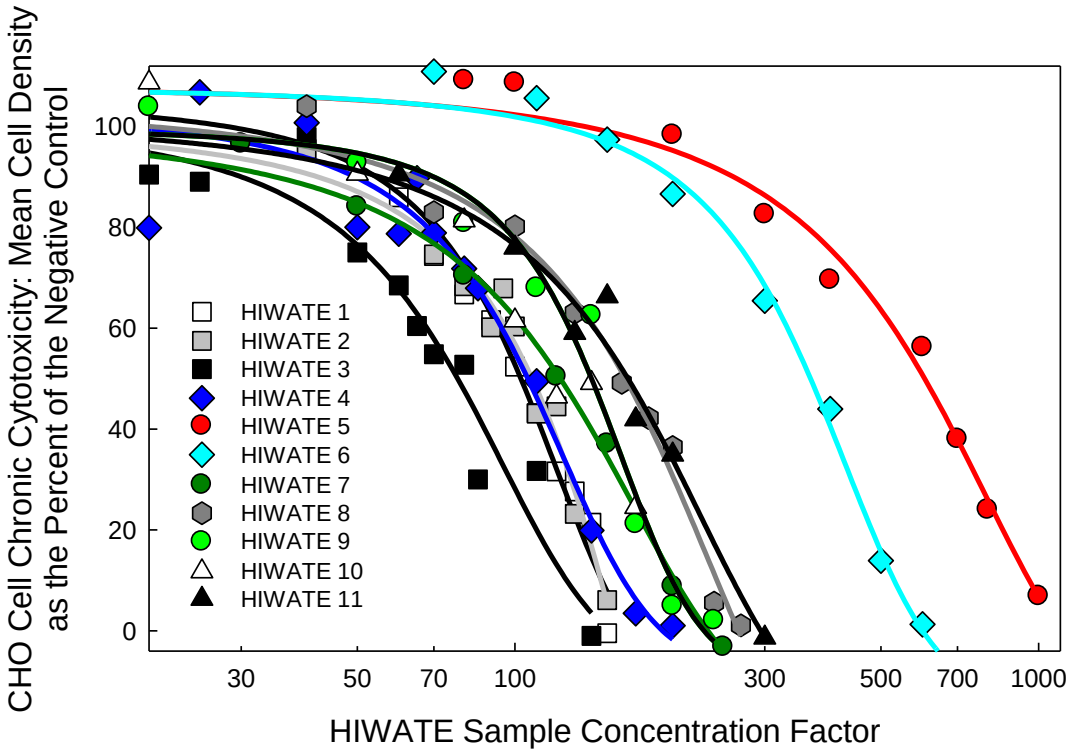


Figure 5.4. Log-linear plot of the concentration-response curves of 11 HIWATE samples illustrating CHO cell chronic (72-h) cytotoxicity.

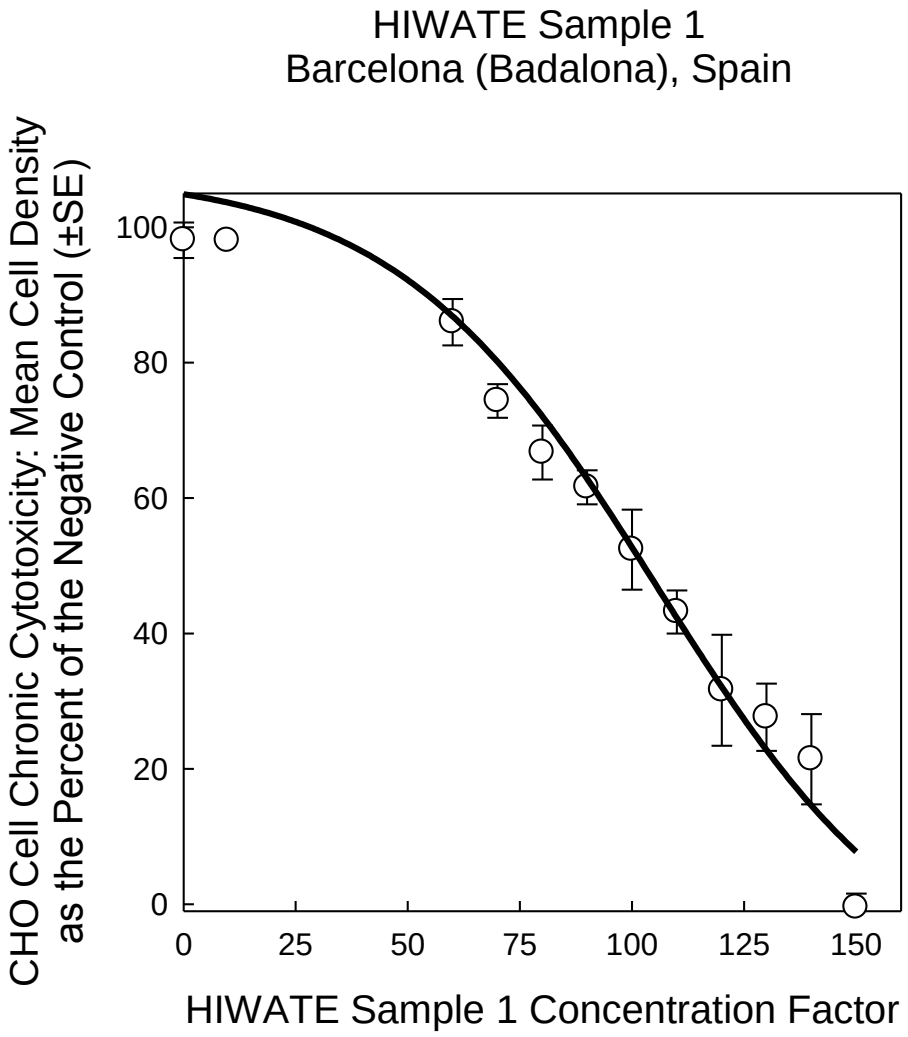


Figure 5.5. HIWATE 1 CHO cell chronic cytotoxicity.

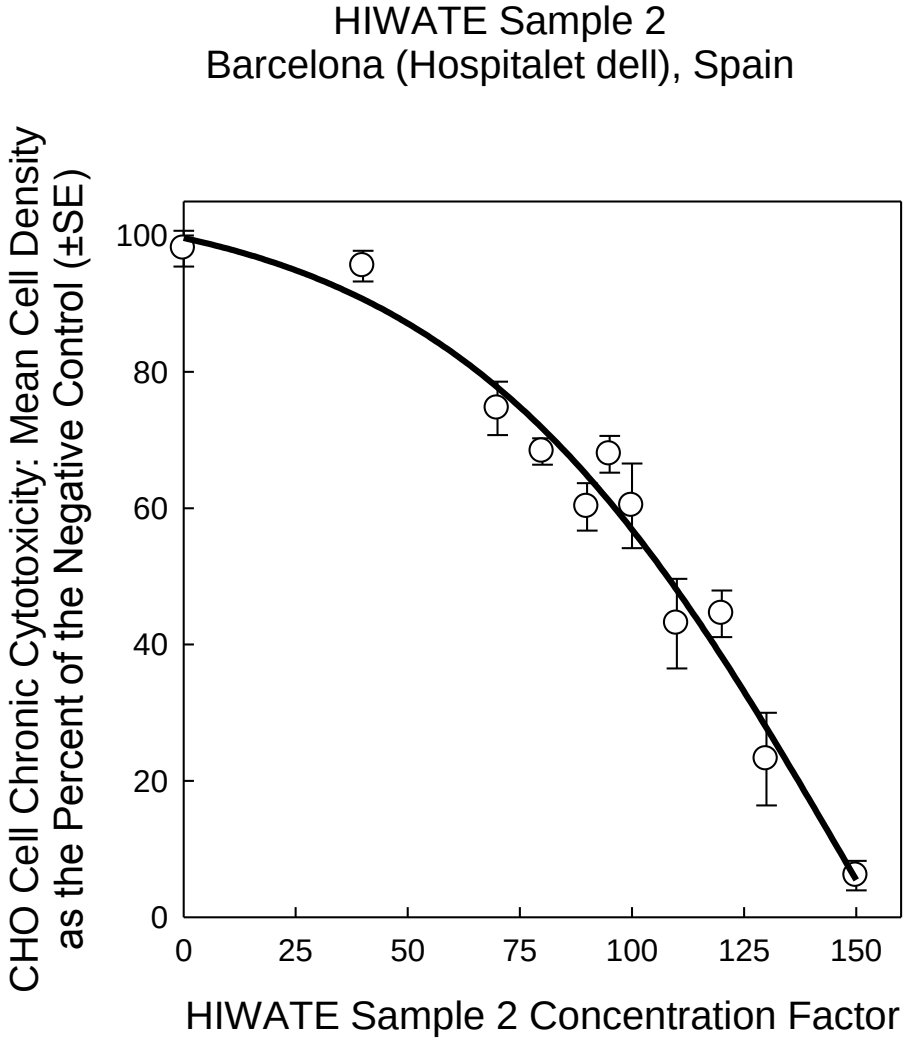


Figure 5.6. HIWATE 2 CHO cell chronic cytotoxicity.

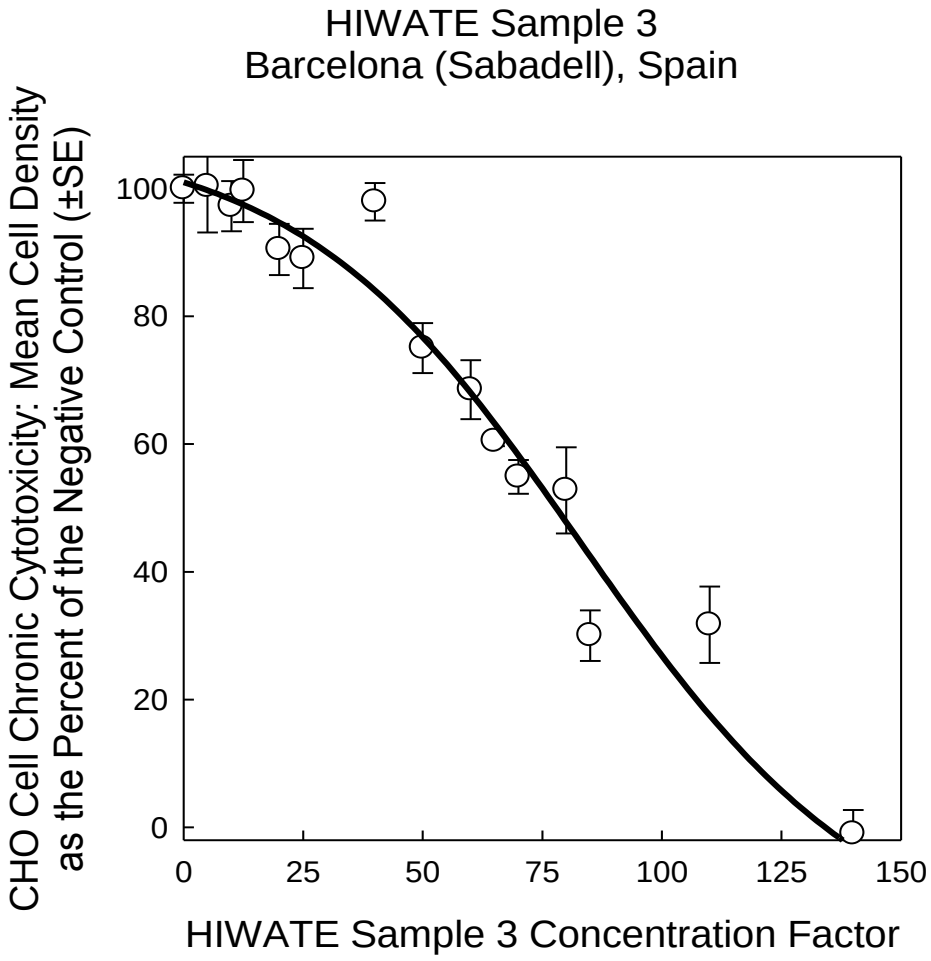


Figure 5.7. HIWATE 3 CHO cell chronic cytotoxicity.

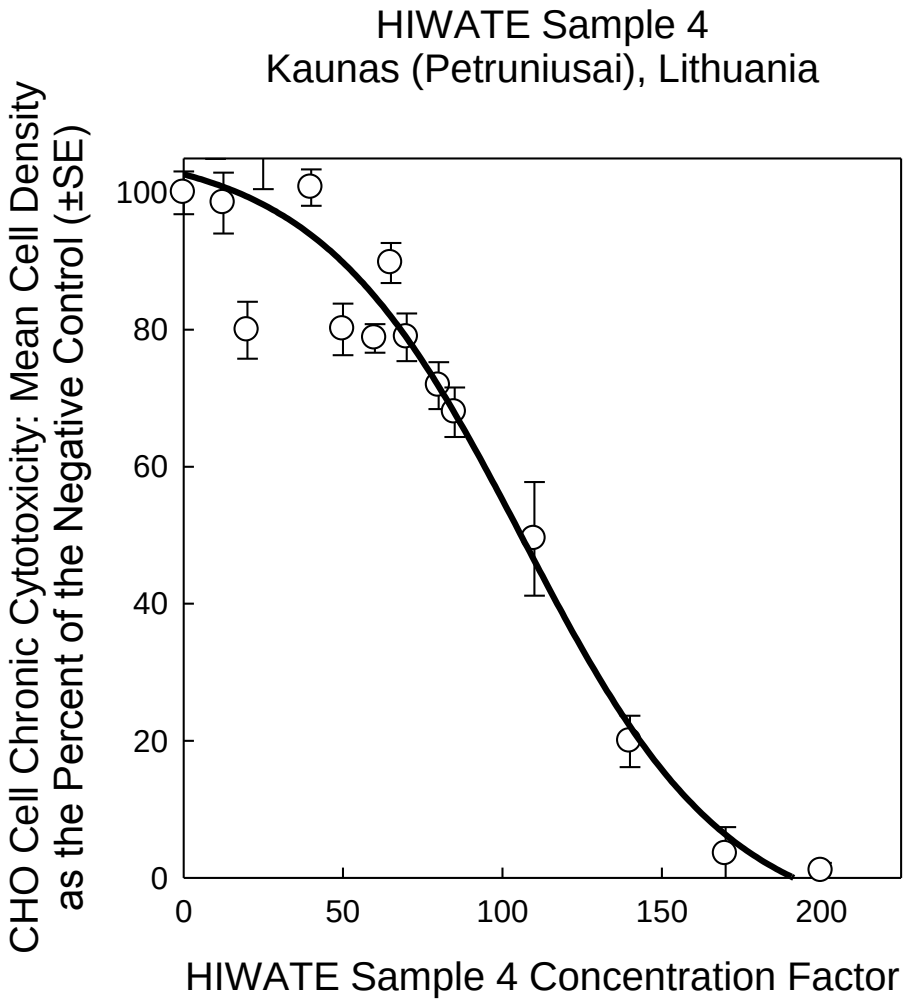


Figure 5.8. HIWATE 4 CHO cell chronic cytotoxicity.

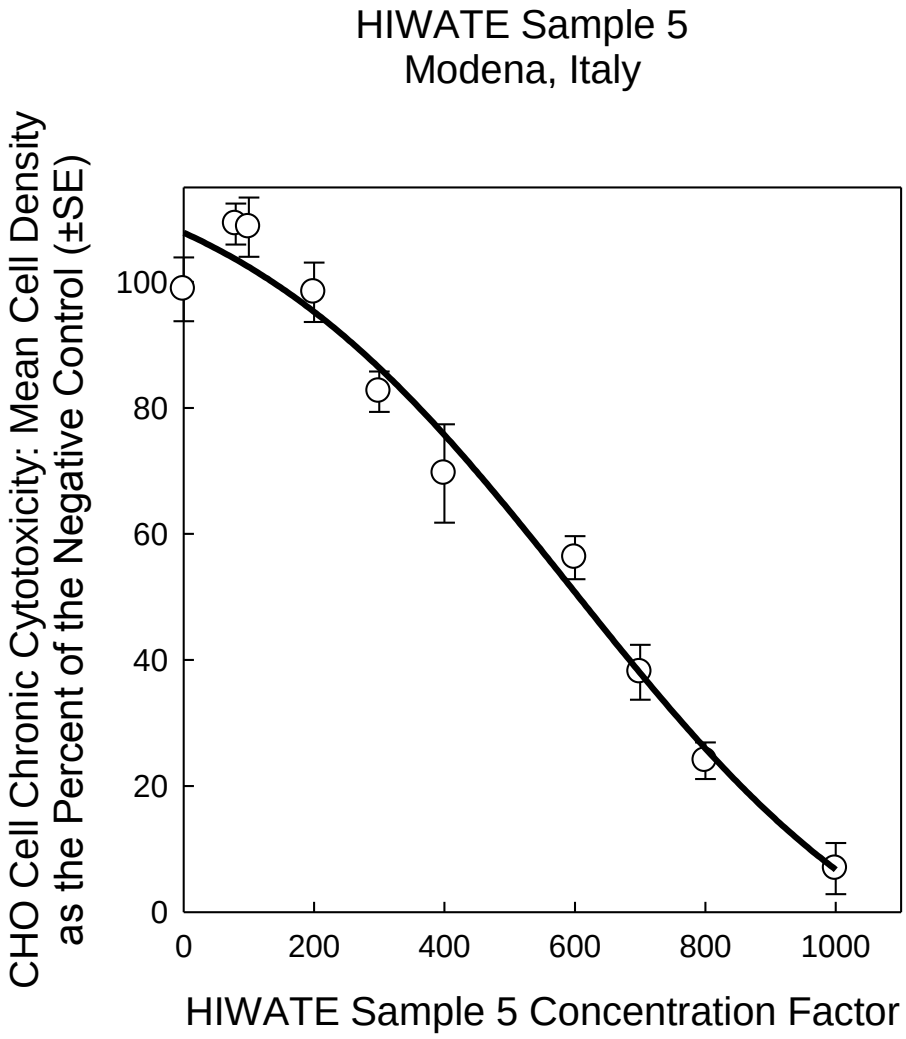


Figure 5.9. HIWATE 5 CHO cell chronic cytotoxicity.

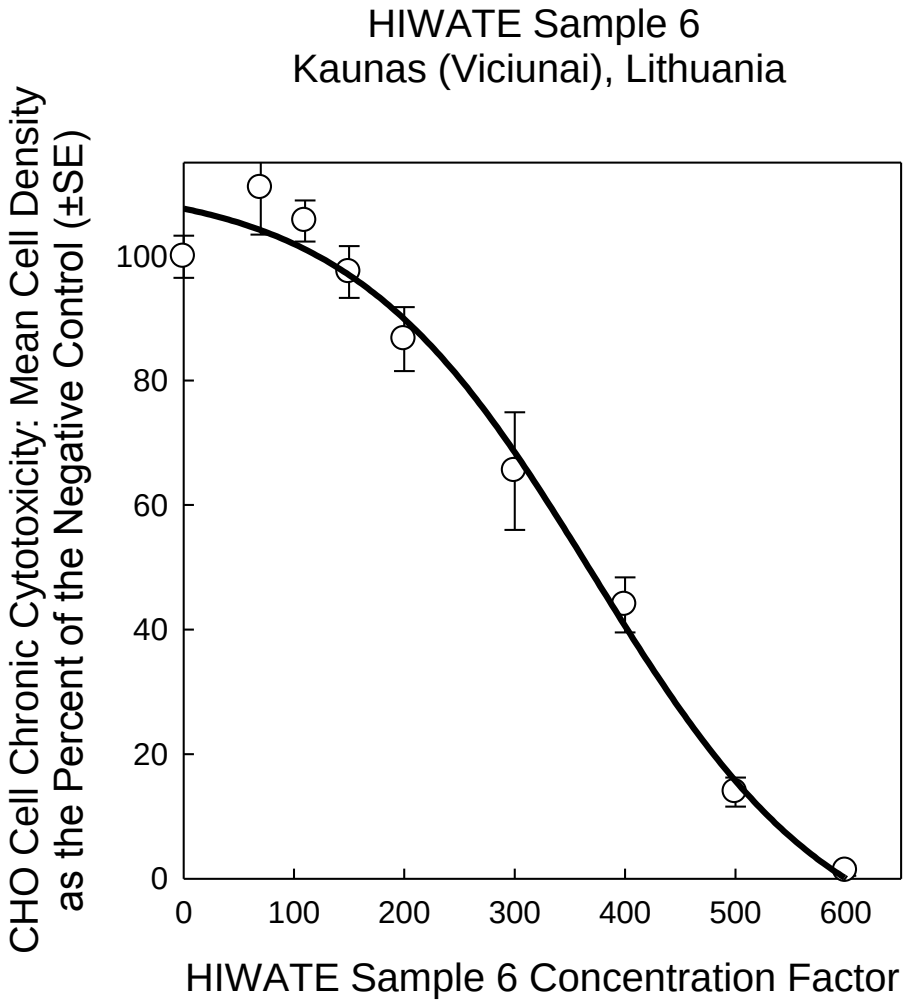


Figure 5.10. HIWATE 6 CHO cell chronic cytotoxicity.

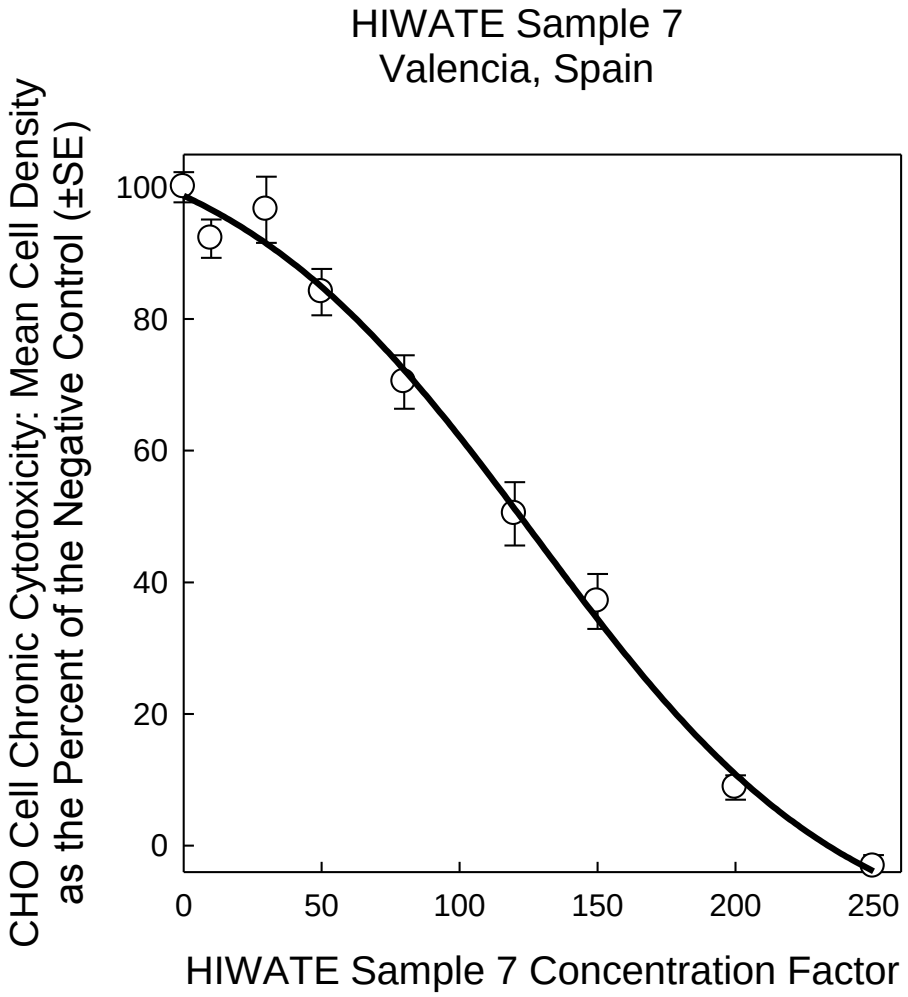


Figure 5.11. HIWATE 7 CHO cell chronic cytotoxicity.

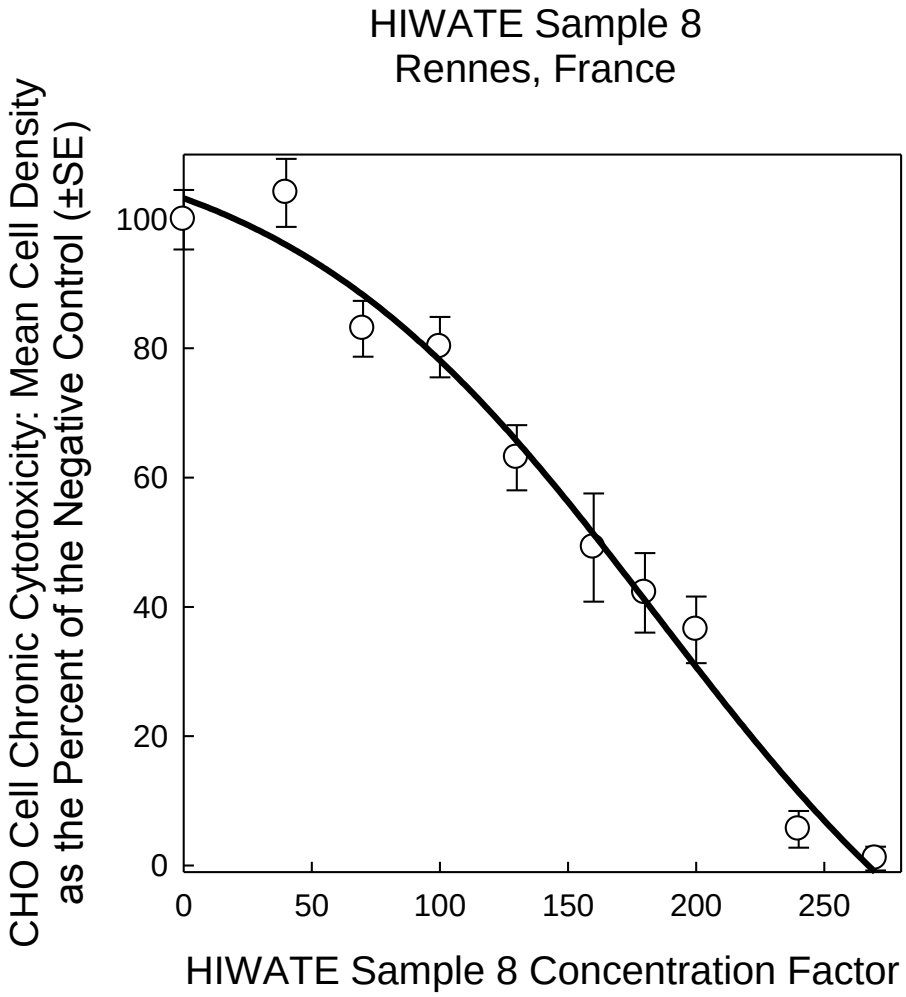


Figure 5.12. HIWATE 8 CHO cell chronic cytotoxicity.

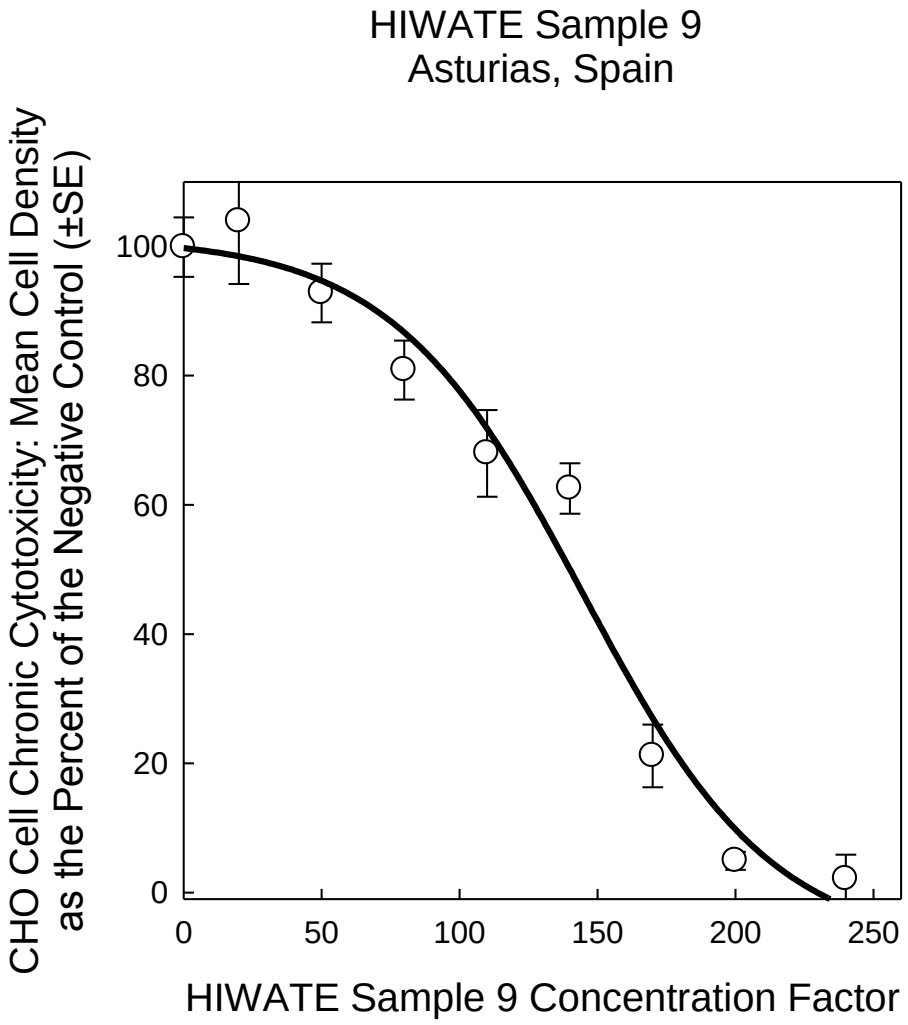


Figure 5.13. HIWATE 9 CHO cell chronic cytotoxicity.

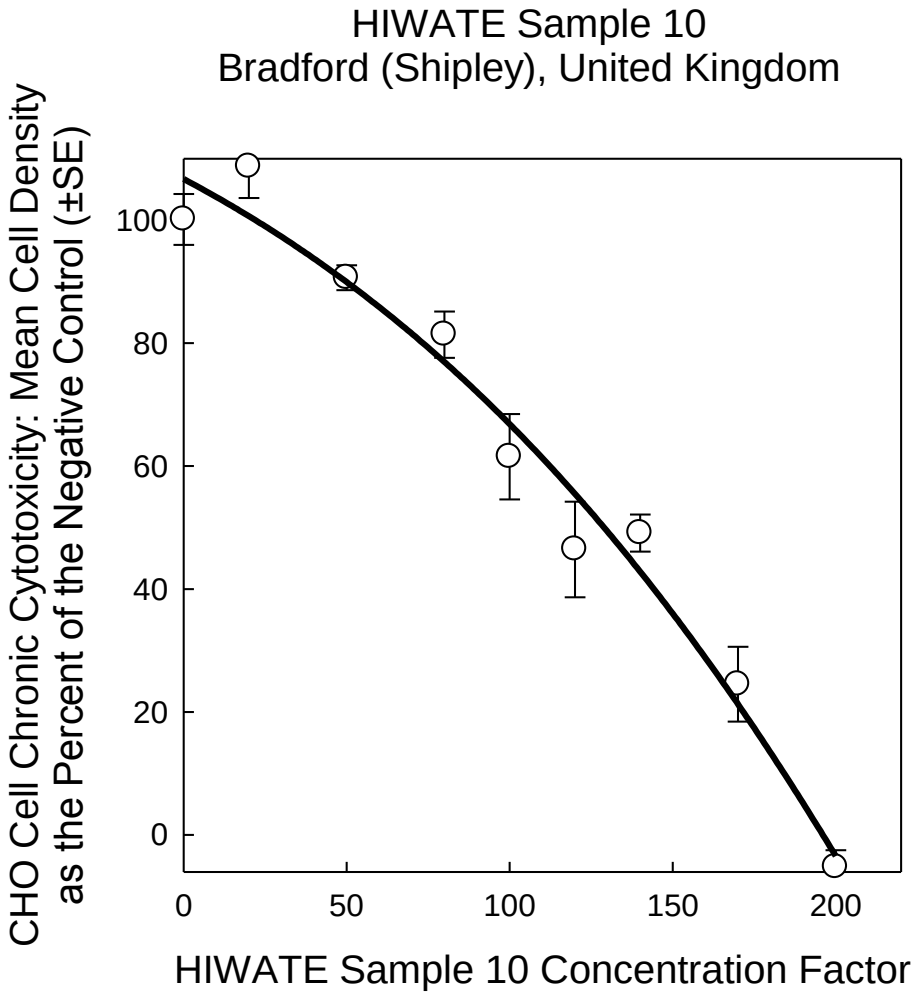


Figure 5.14. HIWATE 10 CHO cell chronic cytotoxicity.

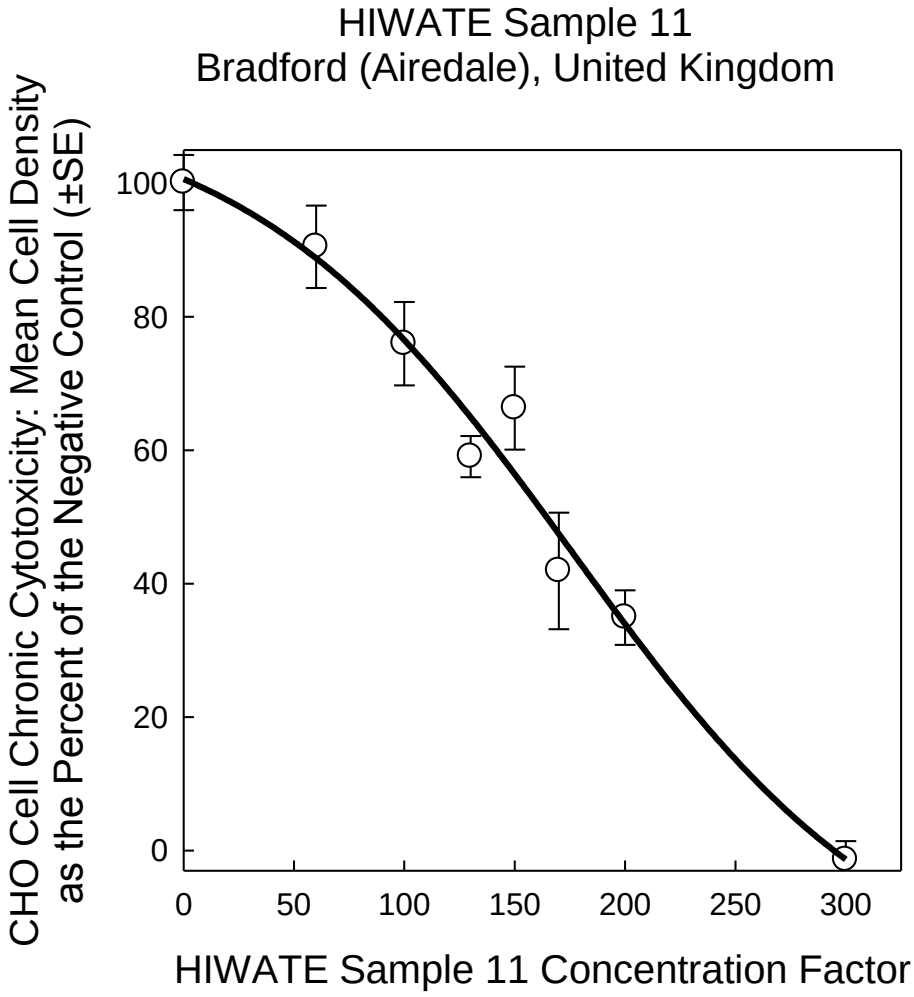


Figure 5.15. HIWATE 11 CHO cell chronic cytotoxicity.

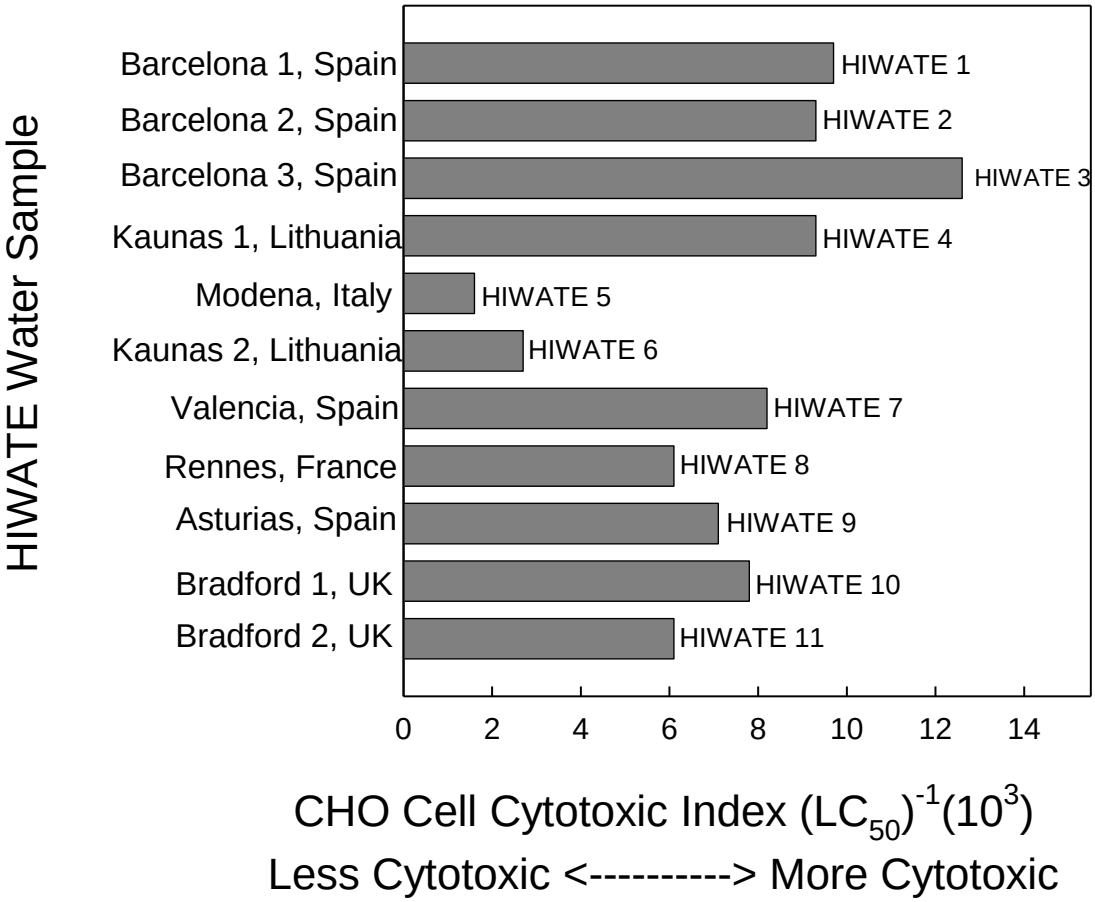


Figure 5.16. The distributions of the CHO cell cytotoxic index values for each HIWATE sample.

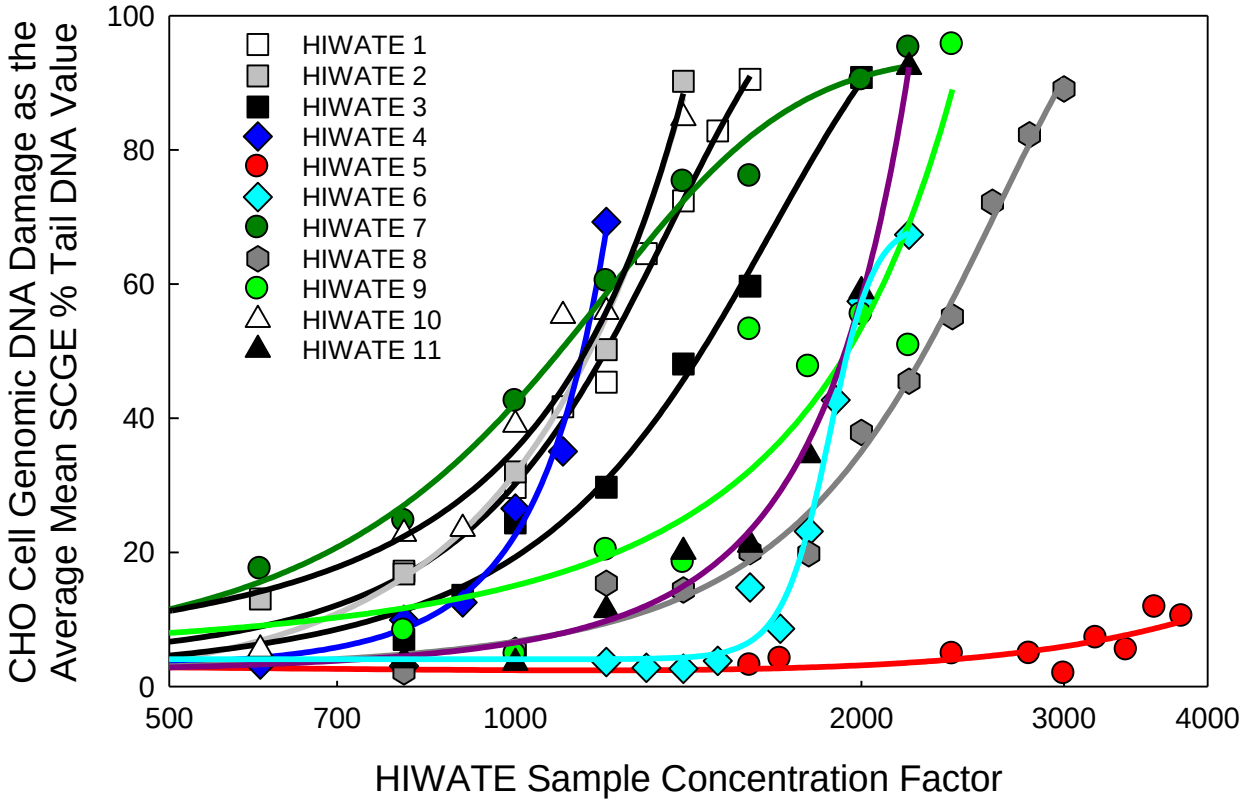


Figure 5.17. Log-linear plot of the concentration-response curves of 11 HIWATE samples illustrating CHO cell acute (4h exposure) genotoxicity.

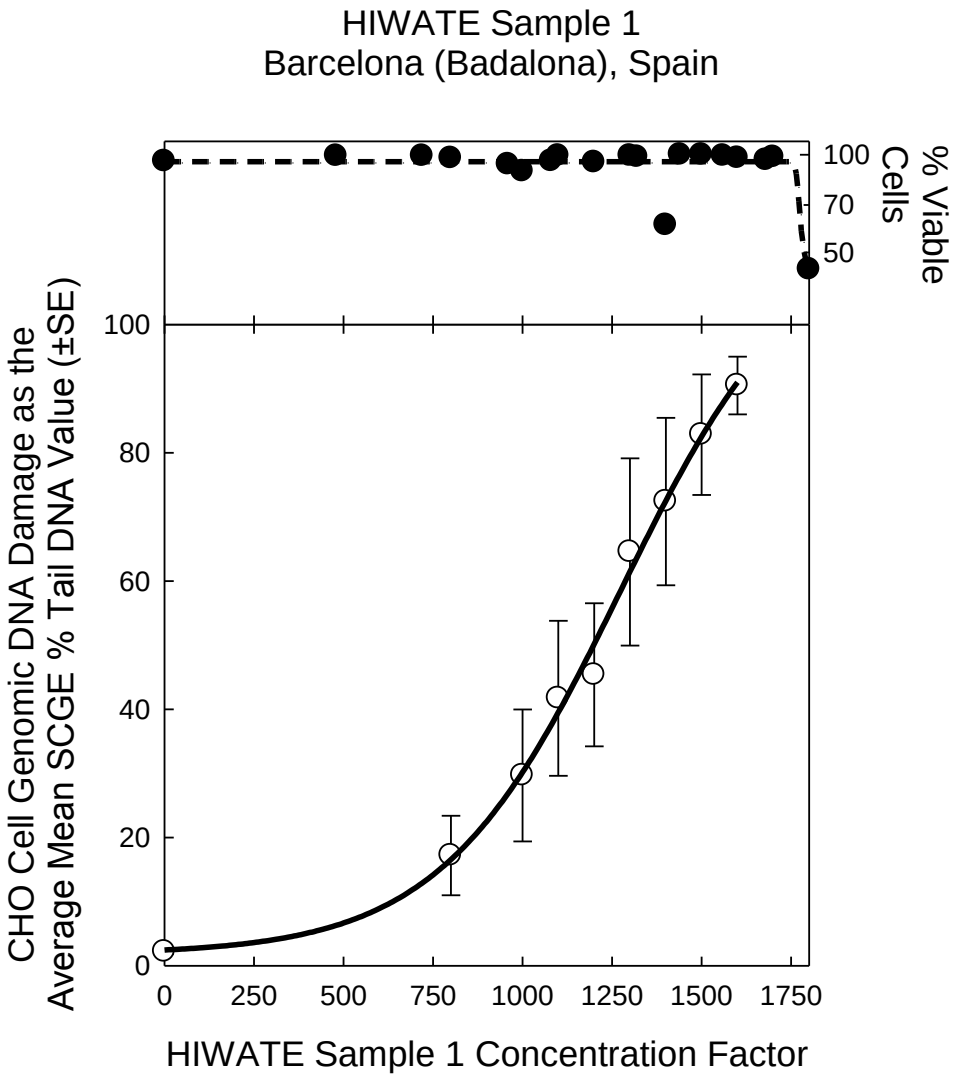


Figure 5.18. HIWATE 1 CHO cell genotoxicity.

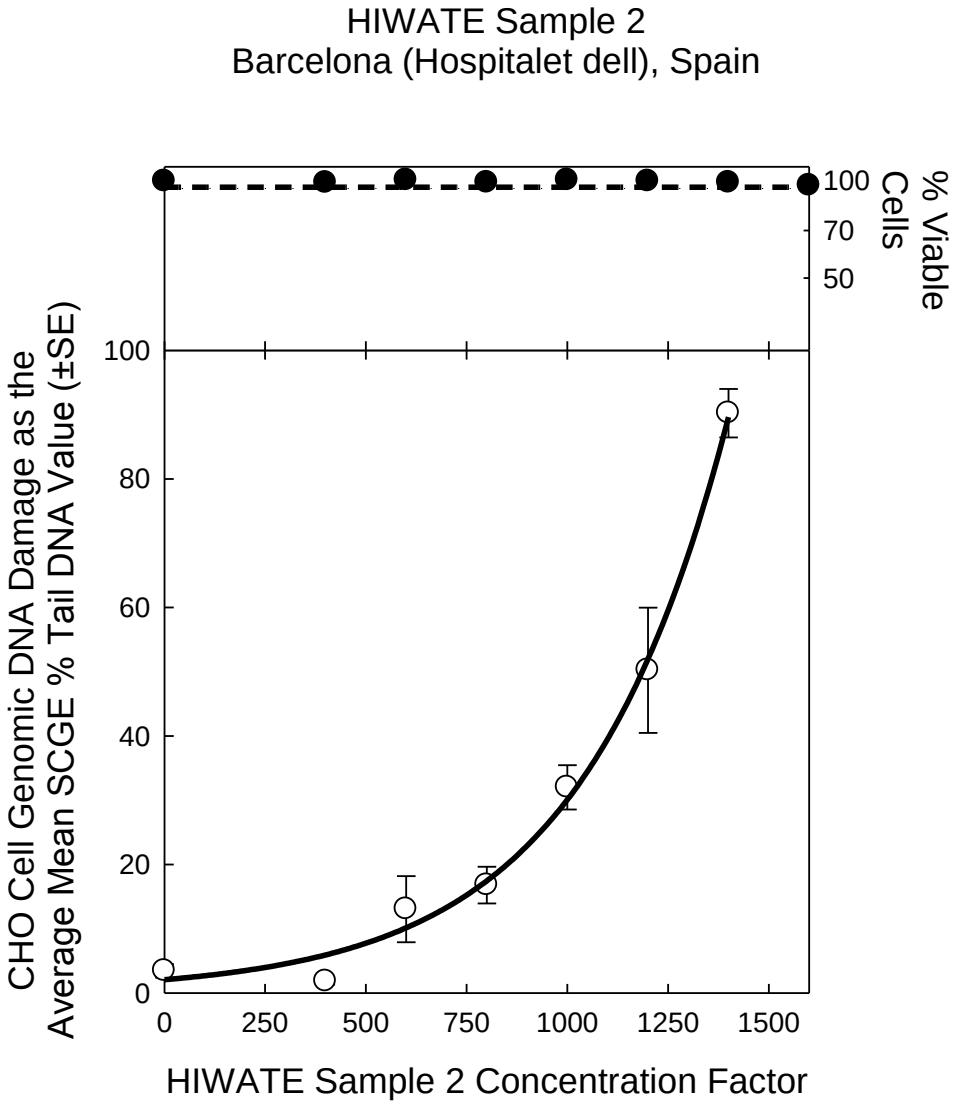


Figure 5.19. HIWATE 2 CHO cell genotoxicity.

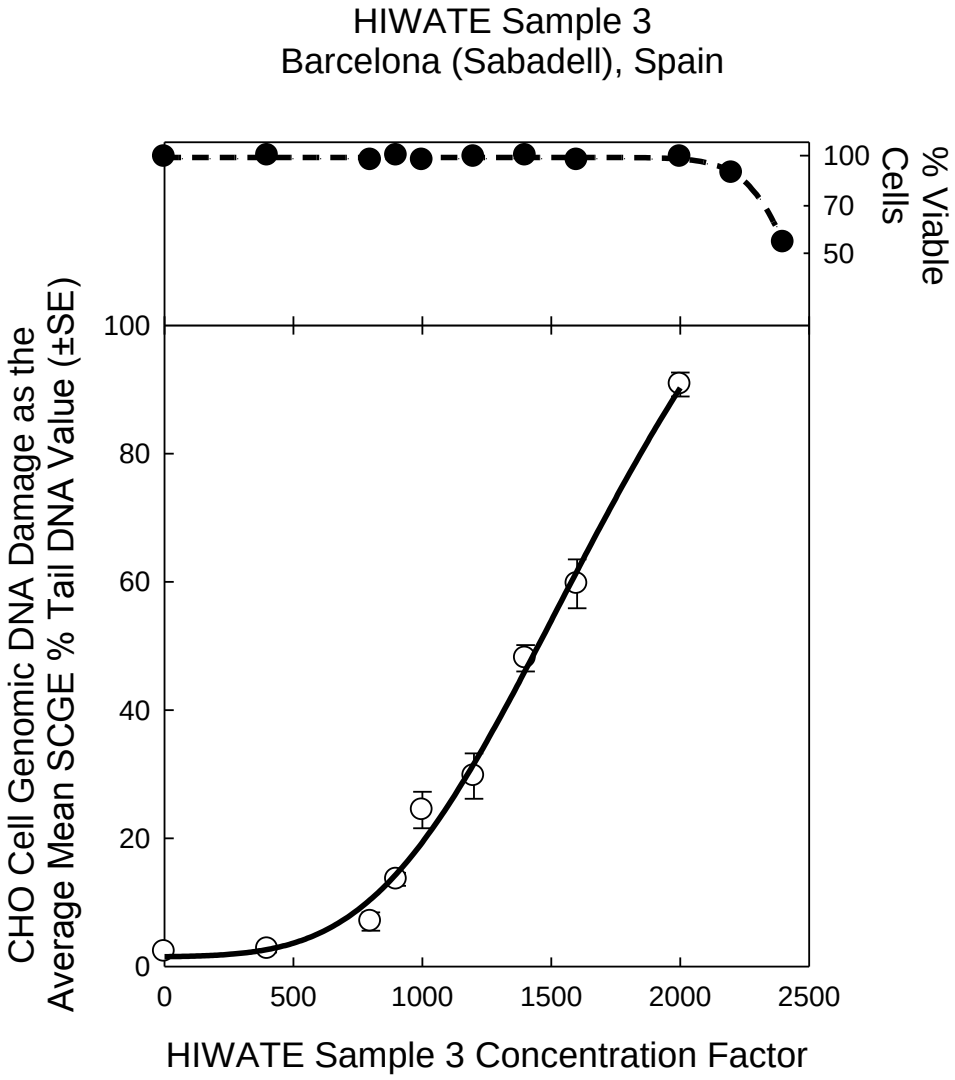


Figure 5.20. HIWATE 3 CHO cell genotoxicity.

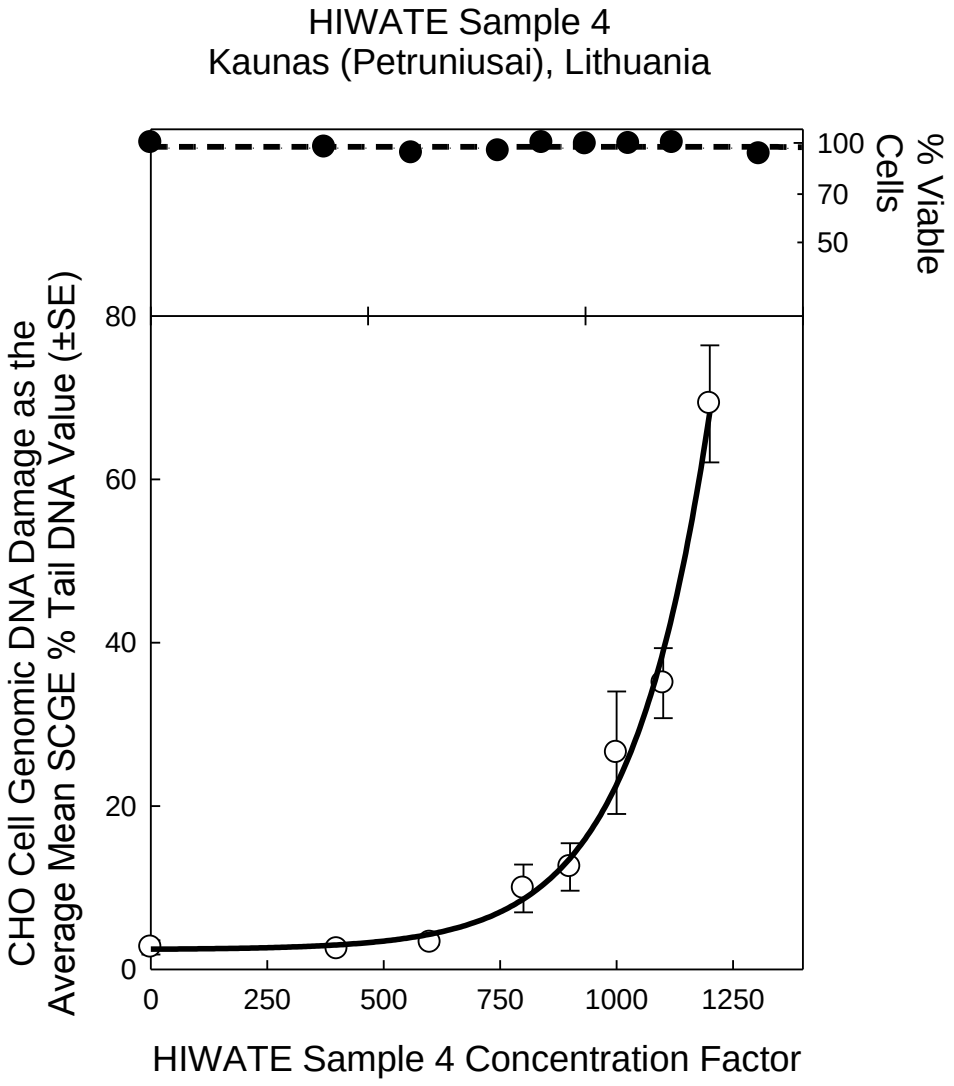


Figure 5.21. HIWATE 4 CHO cell genotoxicity.

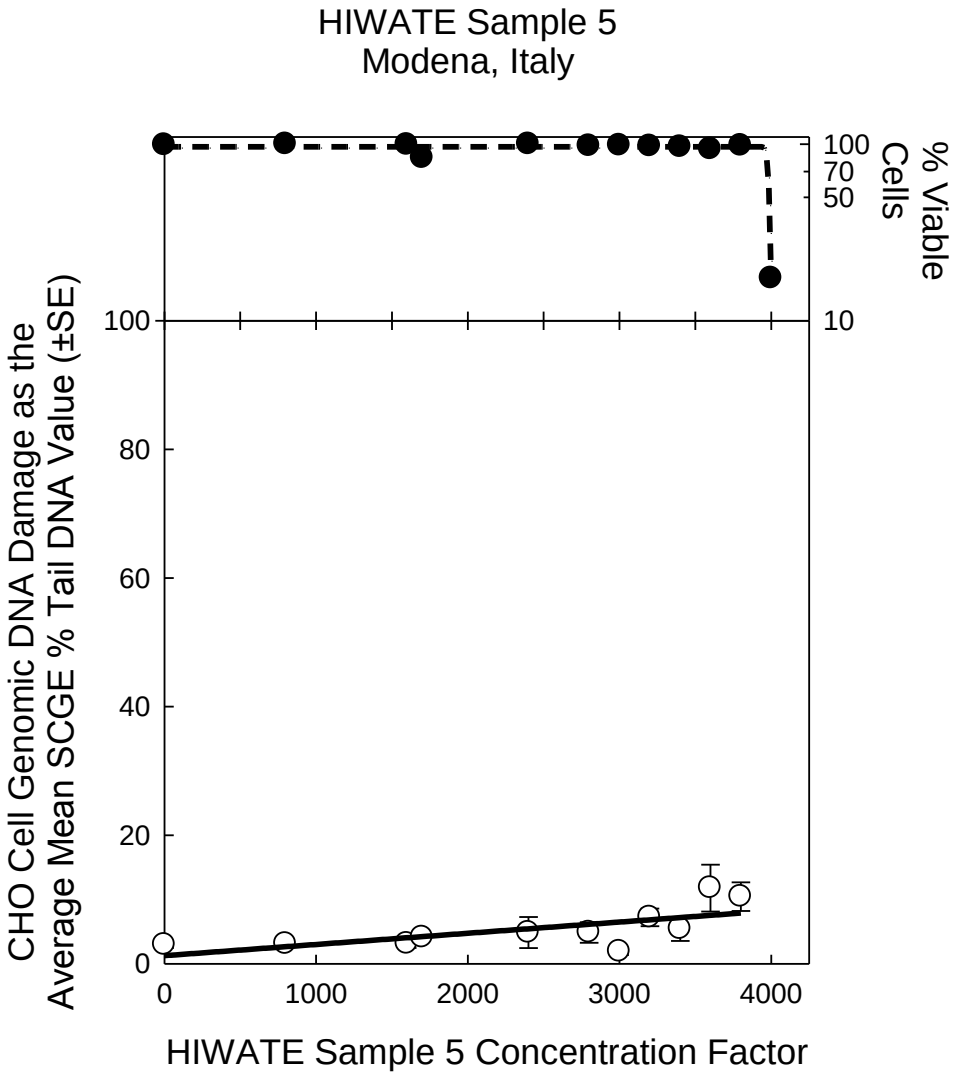


Figure 5.22. HIWATE 5 CHO cell genotoxicity.

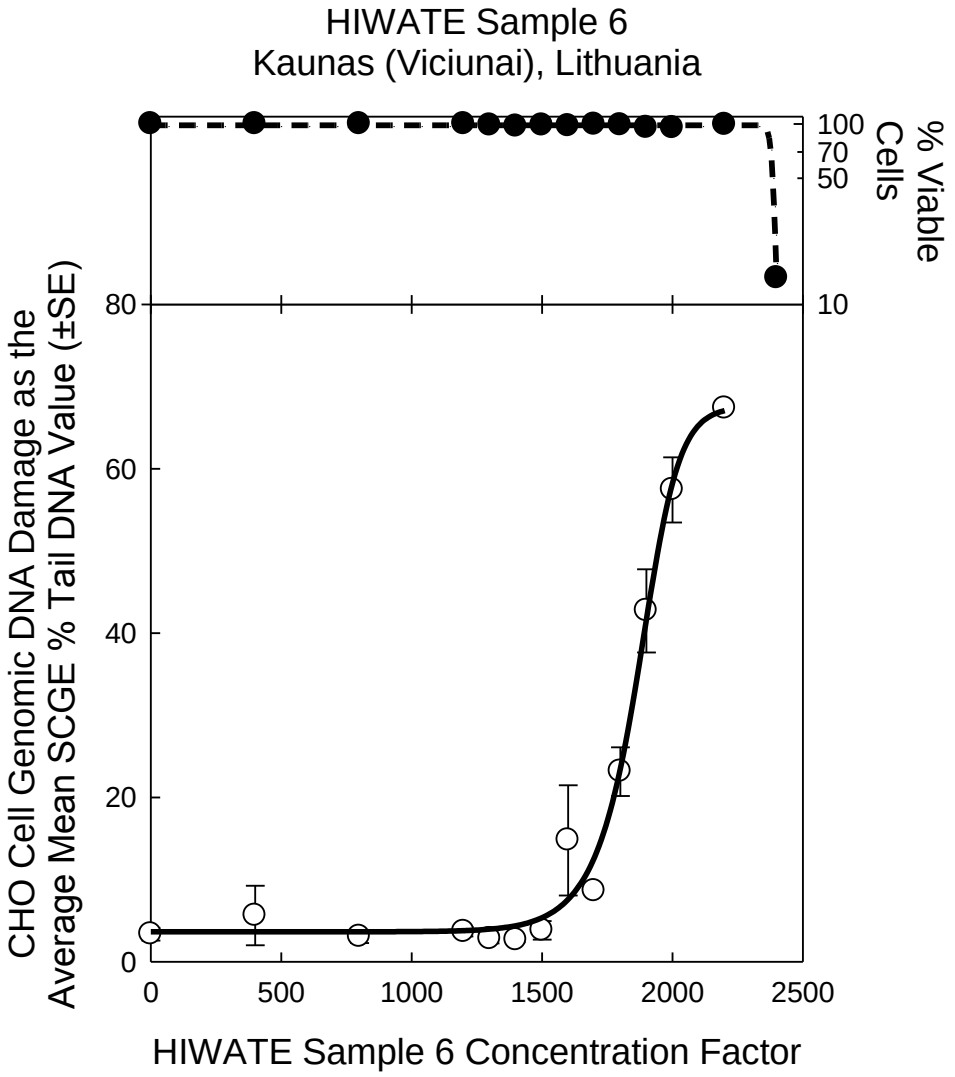


Figure 5.23. HIWATE 6 CHO cell genotoxicity.

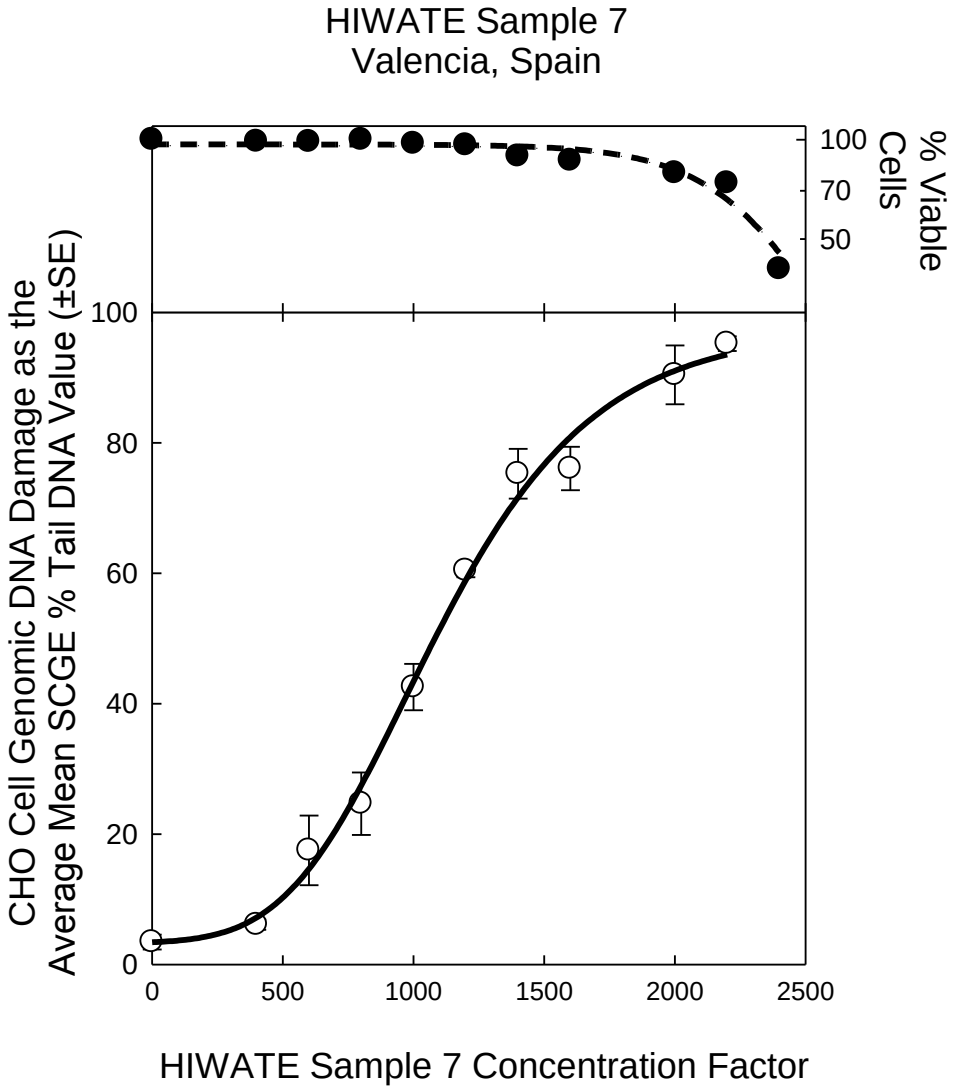


Figure 5.24. HIWATE 7 CHO cell genotoxicity.

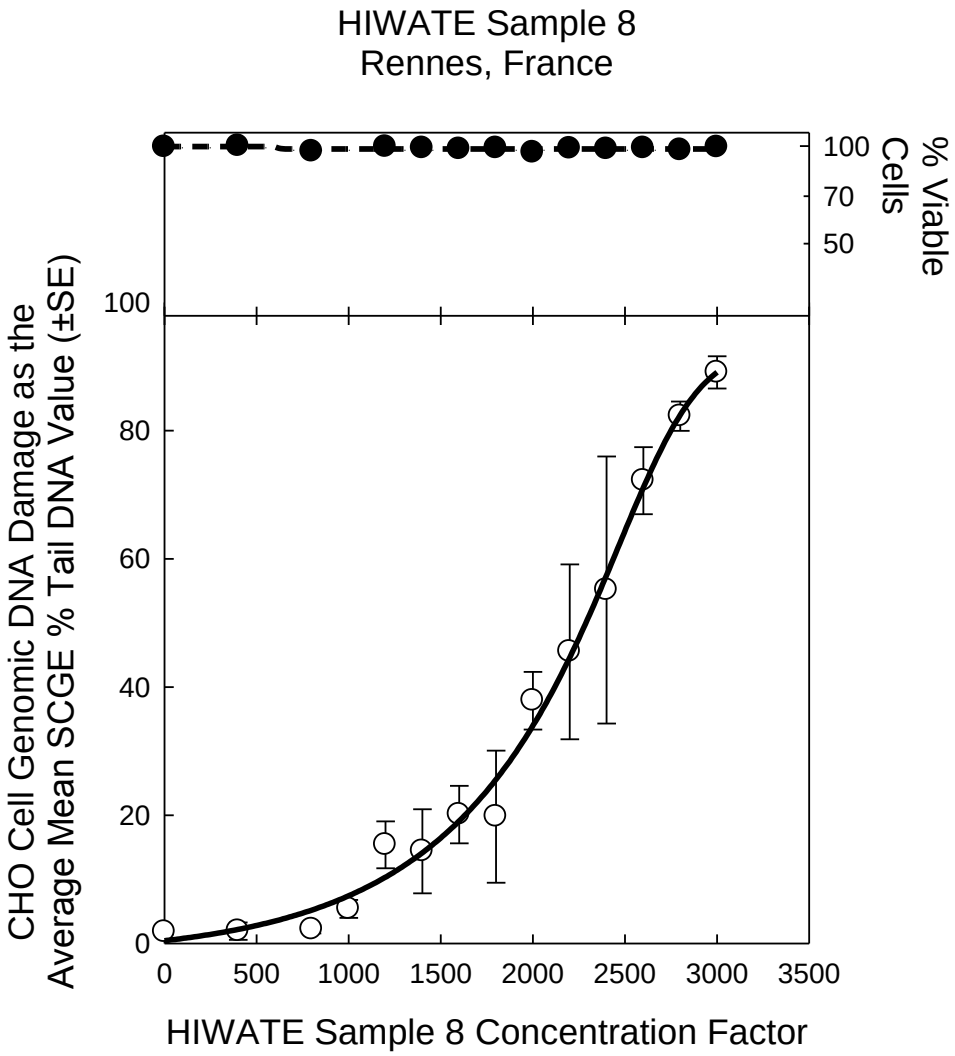


Figure 5.25. HIWATE 8 CHO cell genotoxicity.

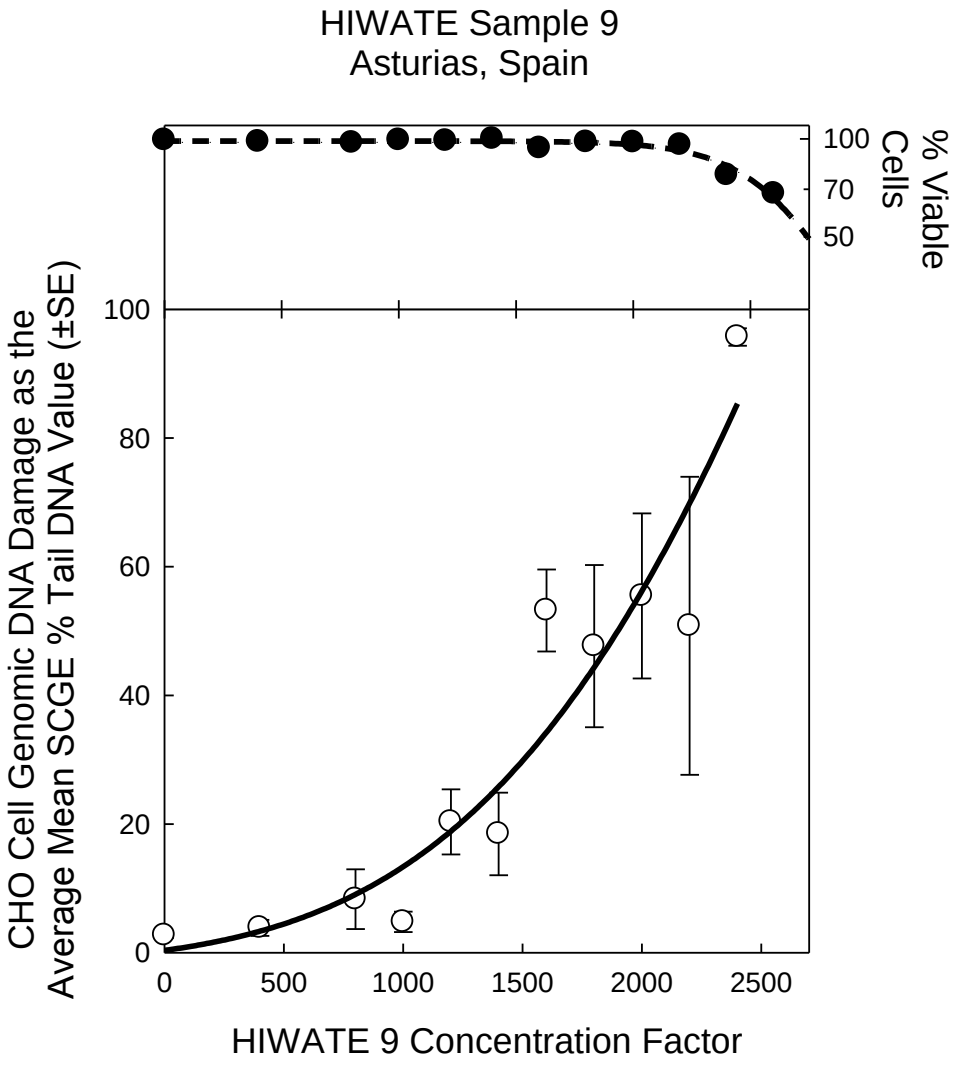


Figure 5.26. HIWATE 9 CHO cell genotoxicity.

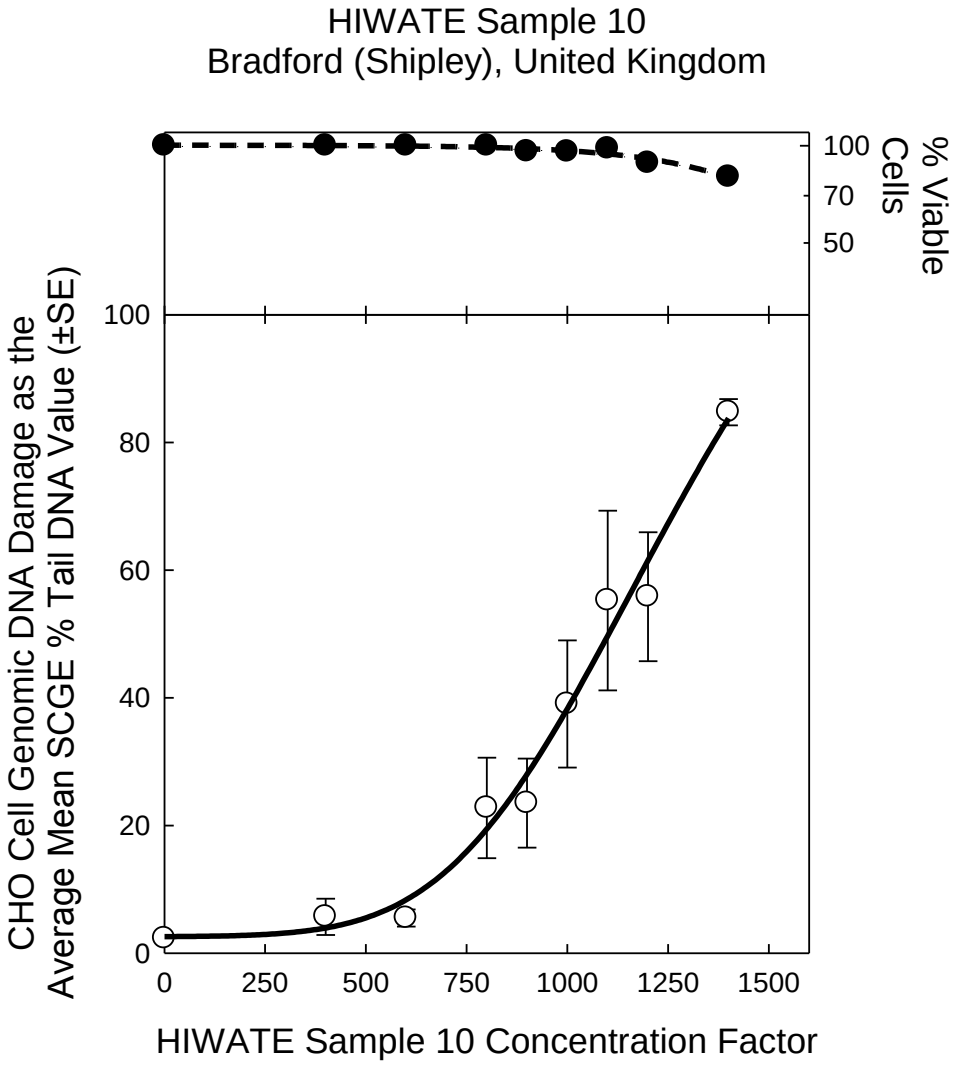


Figure 5.27. HIWATE 10 CHO cell genotoxicity.

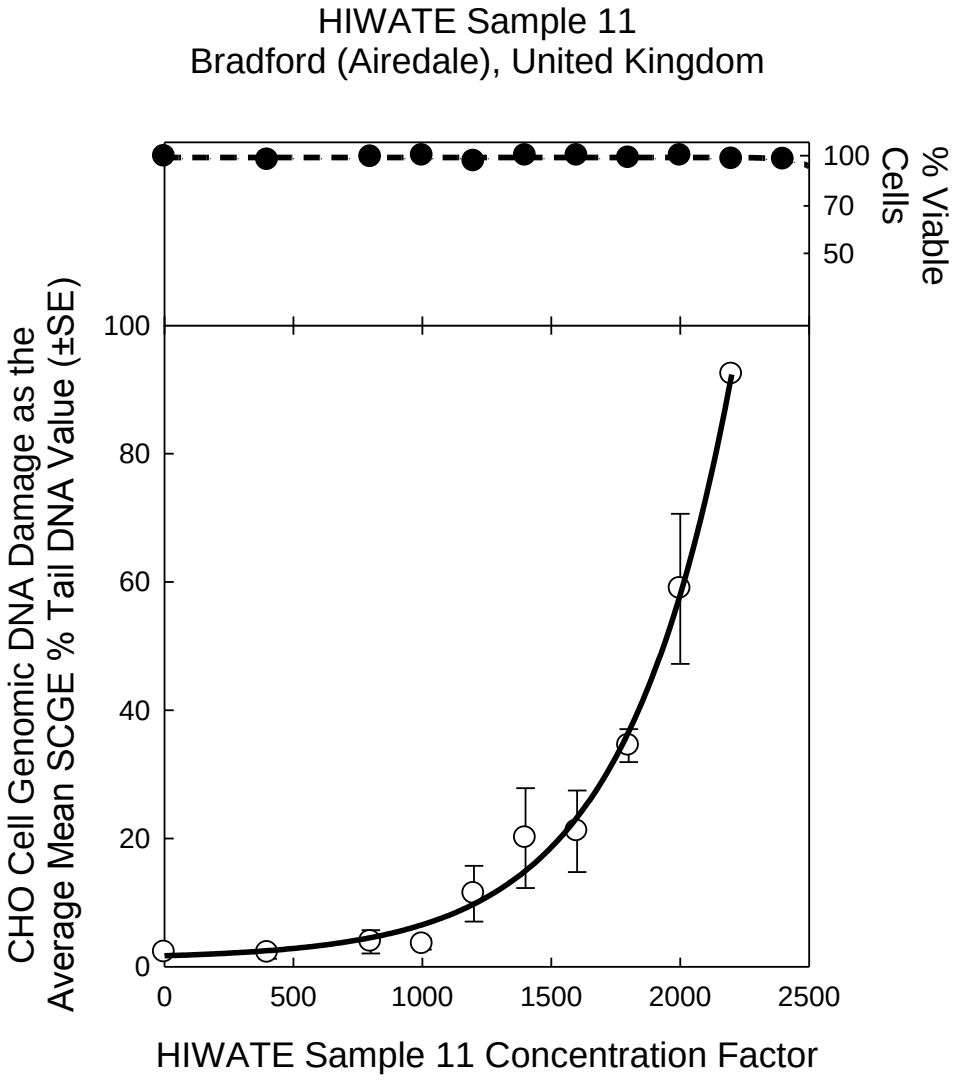


Figure 5.28. HIWATE 11 CHO cell genotoxicity.

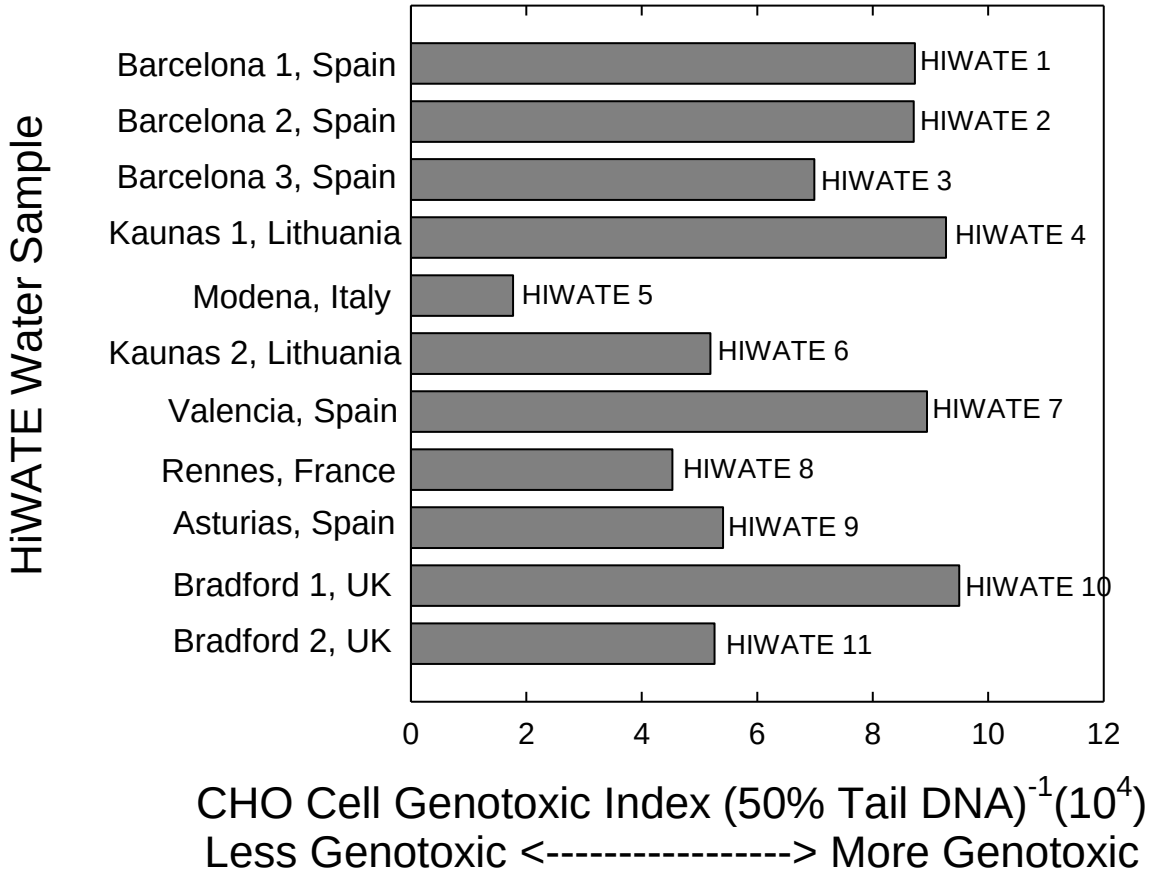


Figure 5.29. The distributions of the CHO cell genotoxic index values for each HIWATE sample.

CHAPTER 6

THE IMPACT OF X-RAY CONTRAST AGENTS ON FORMATION AND TOXICITY OF DISINFECTION BY-PRODUCTS IN DRINKING WATER

PREFACE

This work is a part of an international collaborative study involving research on analytical chemistry and toxicology. Dr. T. Ternes (Bundesanstalt für Gewässerkunde, Koblenz Germany), Dr. S. Duirk (University of Akron) and Dr. S. Richardson (University of South Carolina) are conducting the analytical chemistry experiments on the DBP formation kinetics and DBP identification.

6.1. INTRODUCTION

Drinking water disinfection by-products (DBPs) are formed through the reaction between disinfectants and natural organic matter (NOM). The levels and distribution of DBPs highly depend on the source water characteristics such as pH, temperature, and other contaminants present in the raw water. Emerging environmental contaminants which can react with disinfectants to form contaminant DBPs include consumer products, pharmaceuticals, pesticides, etc. [1, 2]. With all source water conditions being equal, the type of disinfectant generates different levels of total organic halide (TOX) as well as significant differences in the resulting distribution of DBP classes [3, 4]. For example, when source waters were collected and

treated with different disinfectants, chlorination produced a higher level of total organic chlorine (TOCl) and total organic bromine (TOBr) than chloramination and chlorine dioxide treatment in the presence of bromide. Pre-ozonation decreased the formation of TOX during post-chlorination [4].

Iodinated X-ray contrast media (ICM) are widely used at medical centers to enhance the visibility of fluids or structures within the body such as the blood vessels or gastrointestinal tract for medical imaging. Types of ICM vary by their osmolarity, viscosity, and absolute iodine content. ICM have a triiodobenzoic acid unit as their basic structure and their molecular weights vary (600 – 850 Da) by the type of side chains including carboxyl, hydroxyl, and amide functional groups (Figure 6.1). The worldwide consumption of ICM is around 3.5×10^6 kg/year, and a single application can be up to 200g/d. After application, 95% of unmetabolized ICM are eliminated through urine and feces within 24 hours [5]. While ICM are generally stable in water, several biological transformation products (TPs) of these ICM were identified (Figure 6.2) [6]. TPs are formed in wastewater treatment and enter source waters [6]. Incomplete removal of ICM in wastewater treatment plants could lead to an elevation of ICM concentrations in streams and rivers [1, 7-10]. ICM were also found in groundwater [11].

The ICM in source waters may act as potential DBP precursors and react with oxidizing disinfectants to form highly toxic DBPs such as iodinated DBPs (iodo-DBPs) and higher molecular weight DBPs of unknown toxicity. Iodo-DBPs, including iodinated trihalomethanes (iodo-THMs) and iodinated acids (iodo-acids) are commonly found in chlorinated and chloraminated drinking water in the U.S. and Canada [12]. Iodo-DBPs are highly cytotoxic and

genotoxic, and to date, iodoacetic acid is known to be the most genotoxic individual DBP among iodo-DBPs [13]. Naturally occurring iodide (I^-) can be oxidized by disinfectants (chlorine, chloramine, or ozone) to hypoiodous acid (HOI), which can react with NOM or be further oxidized to an inactive iodate (IO_3^-) [14]. In a comparison study, chloramine did not further oxidize hypoiodous acid to iodate, while chlorine and ozone transformed iodide to iodate within seconds to minutes (Figure 6.3) [14]. Therefore, the probability of the iodo-DBPs formation during drinking water disinfection increases in the order of chloramine > chlorine > ozone.

ICM are primary contributors to the TOX burden in clinical wastewater and play a major role as a source of absorbable organic iodine in wastewater [15]. Duirk *et al.* analyzed the source waters for ten of the original 23 cities involved in the iodo-DBP occurrence study [12] and found four ICM including Iopamidol, Iopromide, Iohexol, and Diatrizoate [6]. In this study, Iopamidol was detected most frequently (six of the ten plants) with a maximum concentration of 2.7 μg/L. The chemical mechanism involved in the formation of iodo-DBPs by ICM is known to be different from the mechanism involving the naturally occurring iodide. Reactions with Iopamidol appear to involve an initial attack of disinfectants to Iopamidol and release iodine which reacts with NOM to form iodo-DBPs [6]. Duirk *et al.* suggested that ICM can act as an organic iodine source to form iodo-DBPs in chlorinated or chloraminated NOM-containing source waters (Figure 6.4) [6]. Besides the formation of iodo-DBPs, the activated benzene ring and other functional groups attached can react with oxidizing disinfectants and form high molecular DBPs of unknown toxicity.

The overall hypothesis of this study is that oxidizing disinfectants react with non-toxic ICM to form highly toxic iodo-DBPs and higher molecular weight DBPs of unknown toxicity. This is an international collaborative study (U.S. and Germany) involving analytical chemistry and toxicology and both real source waters and isolated NOM from the U.S. and Germany was used in these experiments. We conducted the controlled laboratory reactions of five ICMs (Iopamidol, Diatrizoate, Iopromide, Iomeprol, and Iohexol) with chlorine and chloramine under different conditions including changes in pH, oxidant dose, NOM level, and bromide concentrations to simulate drinking water treatment. Specifically, the German researchers will identify the initial, high molecular weight DBPs formed during the reaction of ICM and their TPs with oxidants. The German group will also determine the kinetic parameters of these reactions. The U.S. researchers will focus on low molecular weight DBPs including iodo-THMs and iodo-acids formed during the reaction of oxidants with ICM and their TPs. The *in vitro* mammalian cell cytotoxicity and genotoxicity of the reaction product mixtures and of individual identified iodo-DBPs will be measured to determine which ICM and reaction conditions give rise to toxic by-products. This study will elucidate the overall mechanisms of the formation of iodo-DBPs and any other DBPs identified in this research from reactions with ICM and their TPs, and a kinetic model will be developed to predict the formation of iodo-DBPs.

This chapter focuses on the results from the analytical biological studies on the cytotoxicity and genotoxicity of the reaction product mixtures from two experimental designs. The specific objectives of this chapter were to: i) disinfect the source waters from Akron (OH) with chlorine or chloramine with or without Iopamidol, extract and concentrate the organic fraction and determine the relative *in vitro* chronic cytotoxicity and acute genotoxicity in

mammalian cells for each sample, ii) disinfect the source waters from Akron (OH) with chlorine with or without four ICM (Iopromide, Iohexol, Diatrizoate, Iomeprol), extract and concentrate the organic fraction and determine the relative *in vitro* chronic cytotoxicity and acute genotoxicity in mammalian cells for each sample, and iii) analyze for the impact of disinfectant types and individual ICM on overall toxicity.

6.2. MATERIALS AND METHODS

6.2.1. Chemicals and Reagents

General reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) and Fisher Scientific Co. (Itasca, IL). Media and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. (Itasca, IL).

6.2.2. Sample Preparation

Two sets of experiments were conducted for this study. Experimental group 1 consisted of the Akron source water (AOH, 20L) with and without Iopamidol and with and without chlorine or monochloramine disinfection. The conditions of each sample are listed in Table 6.1. Experimental group 2 consisted of the AOH (20L) with and without 4 different ICM (Iopromide, Iohexol, Iomeprol and Diatrizoate) with and without chlorine disinfection (Table 6.2). Water samples were extracted and concentrated to 2 mL ethyl acetate for the biological experiments.

XAD 2 and XAD 8 resins were soxhlet-cleaned according to the procedure provided by Dr. Richardson group in U.S. EPA (Athens, GA) [16]. Soxhlet-cleaned resins were kept in baked

glassware in HPLC-grade methanol at 4°C. The water samples were prepared and extracted over XAD resins by Dr. Duirk's laboratory in University of Akron (Akron, OH). The final extracts were prepared in ethyl acetate and were shipped to our laboratory (Urbana, IL). The ethyl acetate was removed over a stream of dry N_2 gas and solvent exchanged into dimethylsulfoxide (DMSO). These samples were stored in Supelco glass vials with Teflon cap liners at −20° C.

6.2.3. Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8 was used for the biological assays [17-19]. CHO cells were maintained on glass culture plates in Ham's F12 medium containing 5% fetal bovine serum (FBS), 1% antibiotics (100 U/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

6.2.4. CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density as a function of the water sample concentrates (WSCs) over a period of approximately 3 cell divisions (72 h). Chronic cytotoxicity to CHO cells was measured using an assay we previously developed for the analysis of DBPs [20]. For each experiment, a series of dilutions were prepared by diluting the WSCs with F12 culture medium on the day of the experiment and rapidly transferred into the well with CHO cells for treatment. These dilution series represent a range of concentration factors for the organics in the original water. Flat-bottom, tissue culture 96-well microplates were employed; 4 replicate wells were prepared for each concentration of a specific extract for each water sample. Eight wells were reserved for the blank control consisting of 200 µL of F12 medium + 5% fetal bovine

serum (FBS). The negative control consisted of 8 wells containing 100 µL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 µL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known concentration of a water sample organic extract for a total of 200 µL. To prevent cross-over contamination between wells due to volatilization of the organic extract, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells, and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 10 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed in tap water, inverted and tapped dry upon paper towels, and 50 µL of dimethyl sulfoxide/methanol (3:1 v/v) was added to each well for 10 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet on a microcomputer connected to the microplate reader. The blank-corrected absorbance value of the negative control (cells with medium only) was set at 100%. The absorbance for each treatment group well was converted into a percentage of the negative control. For each organic extract concentration, 4-8 replicate wells were analyzed per experiment, and the experiments were repeated 2-3 times. A concentration-response curve was generated for each WSC and a regression analysis was conducted to each curve. The LC_{50} values were calculated from each regression analysis, where the LC_{50} represents the WSC concentration factor that induced a 50% reduction in cell density as compared to the concurrent negative control.

6.2.5. Single Cell Gel Electrophoresis Assay

Single cell gel electrophoresis (SCGE) is a molecular genetic assay that quantitatively measures the level of genomic DNA damage induced in individual nuclei of treated cells [21-23]. We employed the microplate SCGE method [24]. The day before treatment, 4×10^4 CHO cells were added to each microplate well in 200 µL of F12 + 5% FBS and incubated. The next day, the cells were washed with Hank's balanced salt solution (HBSS) and treated with a series of concentrations of an organic extract from the WSCs in F12 medium without FBS in a total volume of 25 µL for 4 h at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal™. After incubation, the cells were washed 2× with HBSS and harvested with 50 µL of 0.01% trypsin + 53 µM EDTA. The trypsin was inactivated with 70 µL of F12 + FBS. Acute cytotoxicity was measured from a 10 µL aliquot of cell suspension mixed with 10 µL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS) [25]. SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The cellular membranes were removed by an overnight immersion in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) at 4°C. The microgels were placed in an alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were neutralized with Tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis of the WSC

samples, the microgels were hydrated in cold water for 30 min and stained with 65 µL of ethidium bromide (20 µg/mL) for 3 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, 2 microgels were prepared per treatment group. Randomly chosen nuclei (25 per microgel) were analyzed using a charged coupled device camera. A computerized image analysis system (Comet IV, Perspective Instruments, Ltd, Suffolk, UK) was employed to determine the SCGE %Tail DNA value of the nuclei as indices of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. Within each experiment, a negative control, a positive control (3.8 mM ethylmethanesulfonate), and concentration series of an organic extract from WSCs were analyzed concurrently. The experiments were repeated 2-3 times for each WSC. Within each concentration factor range with >70% cell viability, a concentration-response curve was generated for each WSC from repeated experiments, and non-linear regression analysis was conducted. The concentration factor that induces 50% of the genomic DNA to migrate from the nucleus (50%Tail DNA value) was calculated from each regression analysis.

6.2.6. Statistical Analysis

For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to determine if the WSC induced a statistically significant level of cell death at a specific concentration. If a significant F value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was performed to identify the lowest cytotoxic concentration. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha = 0.05$. For the

SCGE assay, the %Tail DNA values are not normally distributed which limits the use of parametric statistics [26]. The mean %Tail DNA value for each microgel was calculated and these values were averaged among all of the microgels for each WSC concentration. A one-way ANOVA test was conducted on these averaged %Tail DNA values [27]. If a significant *F* value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power ≥ 0.8 at $\alpha = 0.05$. A bootstrap statistical approach was used to generate a series of multiple LC_{50} values and %Tail DNA per WSC [28, 29].

6.3. RESULTS AND DISCUSSION

6.3.1. Experimental Group 1: CHO Cell Chronic Cytotoxicity Analyses

The CHO cell cytotoxicity concentration-response curves for the experimental group 1 samples are presented in Figures 6.5-6.10. Table 6.3 presents the LC_{50} concentration factor for each WSC and the lowest concentration factor that induced a significant increase from the negative control. A statistical analysis within each WSC for each cytotoxicity concentration-response curve and an ANOVA test against the negative control is also presented in Table 6.3. Using a bootstrap statistical approach a series of multiple LC_{50} values were generated per WSC; for each LC_{50} value a cytotoxicity index (CTI) value was calculated as $(LC_{50})^{-1}(10^3)$. These dimensionless values were then analyzed using an ANOVA test to determine significant differences among the WSC groups. The mean bootstrap CTI (±SE) are presented in Figure 6.11. From an ANOVA test of the bootstrap mean CTI values, significant differences were resolved among the WSC these differences are presented in Table 6.4.

A small, but significant increase in cytotoxicity was induced by Iopamidol alone as compared to the source water, while no significant difference was observed between Iopamidol and the source water after NH_2Cl disinfection (Table 6.4). The chlorinated source water was significantly more cytotoxic than the source water alone or the source water disinfected with monochloramine. Chlorinated source water plus Iopamidol showed the highest CTI value (CTI = 21.0) followed by the chlorinated source water without Iopamidol (CTI = 18.9). A clear significant increase in cytotoxicity was induced by Iopamidol in chloraminated water (CTI without Iopamidol = 7.73, CTI with Iopamidol = 16.6). Of importance is that the relative Iopamidol-mediated increase in CHO cell cytotoxicity was much greater when NH_2Cl was used as the disinfectant versus chlorine. The relative Iopamidol-mediated percent increase in cytotoxicity over the chlorinated Akron source water was 7.5%. While the relative Iopamidol-mediated cytotoxicity associated with chloramines disinfection was 114%. An explanation of these results may be that, compared to chlorine, chloramines disinfection enhances the generation of HOI and the formation of highly toxic iodinated DBPs [14, 30]. These data demonstrate that Iopamidol enhances the cytotoxicity of disinfected source water. The Iopamidol-mediated increase in CHO cell toxicity was also observed in a previous study using source waters from Athens Clark County, GA, which indicates that the increased cytotoxicity is independent of source water (Athens Clark County, GA versus Akron, OH).

6.3.2. Experimental Group 1: CHO Cell Acute Genotoxicity Analyses

The CHO cell SCGE genotoxicity concentration-response curves for the experimental group 1 samples are presented in Figures 6.12-6.17. Table 6.5 presents a statistical analysis

within each WSC for each genotoxicity concentration-response curve and a test against the negative control. Table 6.5 presents the 50%Tail DNA concentration factor for each WSC and the lowest concentration factor that induced a significant increase over the negative control. Using a bootstrap statistical approach a series of multiple 50%Tail DNA values were generated per WSC; for each 50%Tail DNA value a genotoxicity index (GTI) value was calculated as $(50\%\text{Tail DNA})^{-1}(10^4)$. These dimensionless values were then analyzed using an ANOVA test to determine significant differences among the WSC groups. The mean bootstrap GTI (±SE) are presented in Figure 6.18. From an ANOVA test of the bootstrap mean genotoxicity index values, significant differences were resolved among the WSC; these differences are presented in Table 6.6.

The presence of Iopamidol enhanced the genotoxicity of the source water. No significant difference was observed between the source water and the source water after chloramination (Table 6.6). The chlorinated source water was significantly more genotoxic than the source water alone or the chloraminated source water (Figure 6.18). A small, but significant increase in genotoxicity was induced by Iopamidol alone as compared to the source water. Chlorinated water with Iopamidol was significantly more genotoxic than the chlorinated source water alone. A significant increase in genotoxicity was induced by Iopamidol in chloraminated water but the GTI value for chloraminated water with Iopamidol (GTI = 37.1) was smaller than the GTI value for chlorinated water only (GTI = 44.8). The relative Iopamidol-mediated percent increase in genotoxicity over the chlorinated Akron source water was 66%, while the relative Iopamidol-mediated percent increase in genotoxicity over the chloraminated water was 97%. As with the CHO cytotoxicity data, an explanation of these results may be that chloramines

disinfection enhances the generation of HOI and the formation of highly toxic iodinated DBPs [14, 30]. These data demonstrate that besides affecting cytotoxicity, Iopamidol enhances the genotoxicity of disinfected source water. These results were in agreement with our previously published work and indicate that the Iopamidol-mediated increased genotoxicity was independent of source water [6].

6.3.3. Experimental Group 2: CHO Cell Chronic Cytotoxicity Analyses

The CHO cell cytotoxicity concentration-response curves for the experimental group 2 samples are presented in Figures 6.19-6.26. Table 6.7 presents a statistical analysis within each experimental group 2 water sample concentrate for each cytotoxicity concentration-response curve and an ANOVA test against the negative control. Table 6.7 also presents the LC_{50} concentration factor for each WSC and also the lowest concentration factor that induced a significant increase from the negative control. CTI values for each sample were obtained through the approach described in **section 6.3.1**. The mean bootstrap CTI (±SE) are presented in Figure 6.27. The significant differences in CTI values among the WSCs are presented in Table 6.8.

Cytotoxicity significantly increased for all chlorinated groups compared to their non-chlorinated groups. Based on the CTI values, Iopromide showed the lowest increase (41% higher compared to the non-chlorinated water with Iopromide) while Iohexol showed the highest increase (224% higher compared to the non-chlorinated water with Iohexol) among the four ICM pairs.

6.3.4. Experimental Group 2: CHO Cell Acute Genotoxicity Analyses

The CHO cell SCGE genotoxicity concentration-response curves for the experimental group 2 samples are presented in Figures 6.28-6.35. Table 6.9 presents a statistical analysis within each WSC for each genotoxicity concentration-response curve and a test against the negative control. The 50%Tail DNA concentration factor for each experimental group 2 WSC and the lowest concentration factor that induced a significant increase over the negative control are presented in Table 6.9. GTI values for each sample were obtained through the approach described in section 6.3.2. The mean bootstrap GTI (±SE) are presented in Figure 6.33. Significant differences of GTI values among WSCs are presented in Table 6.10.

The genotoxicity of Iopromide, Iohexol, Diatrizoate or Iomeprol in source water were not significantly different from the source water. Chlorination of ICM containing waters significantly increased the genotoxicity of waters compared to their ICM alone pairs. Only Iohexol enhanced the genotoxicity compared to the chlorinated source water. Interestingly, Iopromide expressed reduced genotoxicity as compared to the chlorinated source water (Figure 6.36).

6.4. CONCLUSION

Iopamidol generated an enhanced level of CHO cell cytotoxicity and genotoxicity in conjunction with either chlorine or chloramines disinfection. These data with the Akron source water are in general agreement with the experiments conducted with Athens, Clark County

source water [6]. These data suggest that Iodo-DBPs, which are more toxic than chlorinated or brominated DBPs, were generated from iodide released from Iopamidol under disinfection conditions leading to enhanced cytotoxicity and genotoxicity [31]. For the other ICMs (Experimental Group 2) Akron source water was compared with and without chlorine disinfection. These data demonstrated that Iopromide, Iohexol, Diatrizoate or Iomeprol alone expressed some cytotoxicity over the control. Also these agents expressed higher cytotoxicity when treated with Cl_2. Using the SCGE assay, only Iohexol expressed the most enhanced genotoxicity compared to the chlorinated source water, while Iopromide reduced the genotoxicity. Of the five X-ray contrast agents evaluated for their mammalian cell cytotoxicity and genotoxicity, Iopamidol in water disinfected with chlorine or chloramines was clearly the most responsive in generating adverse biological responses in the CHO cell assays. For further interpretation, high molecular weight DBPs and low molecular weight DBPs have to be identified and the toxicity of those individual DBPs needs to be determined.

References

1. Carballa, M.; Omil, F.; Lema, J. M.; Llompart, M.; Garcia-Jares, C.; Rodriguez, I.; Gomez, M.; Ternes, T., Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* **2004,** *38*, (12), 2918-2926.
2. Richardson, S. D., Water analysis: emerging contaminants and current issues. *Anal. Chem.* **2009,** *81*, (12), 4645-4677.
3. Zhang, X.; Echigo, S.; Minear, R. A.; Plewa, M. J., Characterization and comparison of disinfection by-products of four major disinfectants. In *Natural Organic Matter and Disinfection By-Products: Characterization and Control in Drinking Water*, Barrett, S. E.; Krasner, S. W.; Amy, G. L., Eds. American Chemical Society: Washington, D.C., 2000; pp 299-314.
4. Hua, G. H.; Reckhow, D. A., Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water Res.* **2007,** *41*, (8), 1667-1678.
5. Perez, S.; Eichhorn, P.; Celiz, M. D.; Aga, D. S., Structural characterization of metabolites of the X-ray contrast agent iopromide in activated sludge using ion trap mass spectrometry. *Anal. Chem.* **2006,** *78*, (6), 1866-1874.
6. Duirk, S. E.; Lindell, C.; Cornelison, C. C.; Kormos, J.; Ternes, T. A.; Attene-Ramos, M.; Osiol, J.; Wagner, E. D.; Plewa, M. J.; Richardson, S. D., Formation of toxic iodinated disinfection by-products from compounds used in medical imaging. *Environ. Sci. Technol.* **2011,** *45*, (16), 6845-6854.
7. Drewes, J. E.; Fox, P.; Jekei, M., Occurrence of iodinated x-ray contrast media in domestic effluents and their fate during indirect potable reuse. *J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng.* **2001,** *36*, (9), 1633-1645.
8. Kormos, J. L.; Schulz, M.; Kohler, H. P.; Ternes, T. A., Biotransformation of selected iodinated X-ray contrast media and characterization of microbial transformation pathways. *Environ. Sci. Technol.* **2010,** *44*, (13), 4998-5007.
9. Putschew, A.; Schittko, S.; Jekel, M., Quantification of triiodinated benzene derivatives and X-ray contrast media in water samples by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* **2001,** *930*, (1-2), 127-134.
10. Schittko, S.; Putschew, A.; Jekel, M., Bank filtration: a suitable process for the removal of iodinated X-ray contrast media? *Water Sci. Technol.* **2004,** *50*, (5), 261-268.
11. Sacher, F.; Lange, F. T.; Brauch, H. J.; Blankenhorn, I., Pharmaceuticals in groundwaters analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *J. Chromatogr. A* **2001,** *938*, (1-2), 199-210.
12. Richardson, S. D.; Fasano, F.; Ellington, J. J.; Crumley, F. G.; Buettner, K. M.; Evans, J. J.; Blount, B. C.; Silva, L. K.; Waite, T. J.; Luther, G. W.; McKague, A. B.; Miltner, R. J.; Wagner, E. D.; Plewa, M. J., Occurrence and mammalian cell toxicity of iodinated disinfection byproducts in drinking water. *Environ. Sci. Technol.* **2008,** *42*, (22), 8330-8338.
13. Plewa, M. J.; Wagner, E. D.; Richardson, S. D.; Thruston, A. D., Jr.; Woo, Y. T.; McKague, A. B., Chemical and biological characterization of newly discovered iodoacid drinking water disinfection byproducts. *Environ. Sci. Technol.* **2004,** *38*, (18), 4713-4722.

14. Bichsel, Y.; von Gunten, U., Oxidation of iodide and hypoiodous acid in the disinfection of natural waters. *Environ. Sci. Technol.* **1999,** *33*, (22), 4040-4045.
15. Kummerer, K.; Erbe, T.; Gartiser, S.; Brinker, L., AOX-emissions from hospitals into municipal waste water. *Chemosphere* **1998,** *36*, (11), 2437-2445.
16. Richardson, S. D., XAD resin extraction of disinfectant by-products drinking water: SOP - RSB-003.1- Revision No. 1. In Environmental Protection Agency: Athens, GA, 2011.
17. Hsie, A. W.; Brimer, P. A.; Mitchell, T. J.; Gosslee, D. G., The dose-response relationship for ultraviolet-light-induced mutations at the hypoxanthine-guanine phosphoribosyltransferase locus in Chinese hamster ovary cells. *Somatic Cell Genet.* **1975,** *1*, (4), 383-389.
18. Tindall, K. R.; Stankowski, L. F., Jr.; Machanoff, R.; Hsie, A. W., Detection of deletion mutations in pSV2gpt-transformed cells. *Mol. Cell Biol.* **1984,** *4*, (7), 1411-1415.
19. Wagner, E. D.; Rayburn, A. L.; Anderson, D.; Plewa, M. J., Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* **1998,** *32*, (4), 360-368.
20. Plewa, M. J.; Wagner, E. D., *Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products*. Water Research Foundation: Denver, CO, 2009; p 134.
21. Fairbairn, D. W.; Olive, P. L.; O'Neill, K. L., The comet assay: a comprehensive review. *Mutat. Res.* **1995,** *339*, (1), 37-59.
22. Rundell, M. S.; Wagner, E. D.; Plewa, M. J., The comet assay: genotoxic damage or nuclear fragmentation? *Environ. Mol. Mutagen.* **2003,** *42*, (2), 61-67.
23. Tice, R. R.; Agurell, E.; Anderson, D.; Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J. C.; Sasaki, Y. F., Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* **2000,** *35*, (3), 206-221.
24. Wagner, E. D.; Plewa, M. J., Microplate-based comet assay. In *The Comet Assay in Toxicology*, Dhawan, A.; Anderson, D., Eds. Royal Society of Chemistry: London, 2009; pp 79-97.
25. Phillips, H. J., Dye exclusion tests for cell viability. In *Tissue Culture: Methods and Applications*, Kruse, P. F.; Patterson, M. J., Eds. Academic Press: New York, 1973; p 406.
26. Box, G. E. P.; Hunter, W. G.; Hunter, J. S., *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*. Wiley & Sons Inc.: New York, NY., 1978.
27. Lovell, D. P.; Omori, T., Statistical issues in the use of the comet assay. *Mutagenesis* **2008,** *23*, (3), 171-182.
28. Efron, B., Better bootstrap confidence intervals *J. Am. Statis. Assoc.* **1987,** *82*, (397), 171-185.
29. Singh, K. X., M. , Bootstrap: A Statistical Method In Rutgers University, 2008; p 18.
30. Bichsel, Y.; von Gunten, U., Formation of iodo-trihalomethanes during disinfection and oxidation of iodide containing waters. *Environ. Sci. Technol.* **2000,** *34*, (13), 2784-2791.

31. Plewa, M. J.; Wagner, E. D.; Muellner, M. G.; Hsu, K. M.; Richardson, S. D., Comparative mammalian cell toxicity of N-DBPs and C-DBPs. In *Occurrence, formation, health effects and control of disinfection by-products in drinking water*, Karanfil, T.; Krasner, S. W.; Westerhoff, P.; Xie, Y., Eds. American Chemical Society: Washington, D.C., 2008; Vol. 995, pp 36-50.

TABLES AND FIGURES

Table 6.1. Experimental group 1: Akron source water with and without Iopamidol (IDOL) and disinfected with either chlorine (Cl) or monochloramine (NH_2Cl).

Sample[a]	Source water	Iopamidol	Disinfection
AOHTOX-DI	DI	NA[b]	NA
AOHTOX-SW	Akron	NA	NA
AOHTOX-IDOL	Akron	5 µM	NA
AOHTOX-CL	Akron	NA	$[Cl_2]_T$ = 100 µM
AOHTOX-CL-IDOL	Akron	5 µM	$[Cl_2]_T$ = 100 µM
AOHTOX-NH_2CL	Akron	NA	$[NH_2Cl]_T$ = 100 µM
AOHTOX-NH_2CL-IDOL	Akron	5 µM	$[NH_2Cl]_T$ = 100 µM

[a] Experiments were performed on 20 L of water sources. Each sample was extracted and concentrated into ethyl acetate with a final volume of 2 mL. [b] NA; not applied.

Table 6.2. Experimental group 2: Akron source water with 4 Iodinated X-ray contrast media (ICM) and with and without chlorine (Cl_2) disinfection.

Sample[a]	ICM	ICM concentration	Disinfection
AOHTOX-Iopromide	Iopromide	5 µM	NA[b]
AOHTOX-CL-Iopromide	Ioprimide	5 µM	$[Cl_2]_T$ = 100 µM
AOHTOX-Iohexol	Iohexol	5 µM	NA
AOHTOX-CL-Iohexol	Iohexol	5 µM	$[Cl_2]_T$ = 100 µM
AOHTOX-Diatrizoate	Diatrizoate	5 µM	NA
AOHTOX-CL-Diatrizoate	Diatrizoate	5 µM	$[Cl_2]_T$ = 100 µM
AOHTOX-Iomeprol	Iomeprol	5 µM	NA
AOHTOX-CL-Iomeprol	Iomeprol	5 µM	$[Cl_2]_T$ = 100 µM

[a] Experiments were performed on 20 L of water sources. Each sample was extracted and concentrated into ethyl acetate with a final volume of 2 mL. [b] NA; not applied.

Table 6.3. Experimental group 1: Comparative CHO cell chronic cytotoxicity of X-ray contrast agent Iopamidol in Akron source water with and without Iopamidol (IDOL) and disinfected with either chlorine (Cl) or monochloramine (NH_2Cl).

Sample	LC_{50} (CF) [a]	r^2 [b]	Lowest Cytotoxic Conc. Factor [c]	ANOVA Test Statistic [d]
AOHTOX-SW	161.3	0.94	75	$F_{10, 50} = 53.5$; $P \leq 0.001$
AOHTOX-CL	53.0	0.94	25	$F_{10, 45} = 141.3$; $P \leq 0.001$
AOHTOX-IDOL	123.7	0.96	75	$F_{9, 48} = 167.8$; $P \leq 0.001$
AOHTOX-CL-IDOL	47.6	0.97	25	$F_{11, 122} = 140.6$; $P \leq 0.001$
AOHTOX-NH_2CL	129.3	0.94	100	$F_{10, 50} = 72.3$; $P \leq 0.001$
AOHTOX-NH_2CL-IDOL	60.3	0.99	25	$F_{10, 125} = 154.3$; $P \leq 0.001$

[a] The LC_{50} value is the fold concentration factor of the WSC sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. [b] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated. [c] Lowest cytotoxic concentration was the lowest concentration factor of the WSC in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 6.4. Experimental group 1: Test for significance among CTI values.

	SW	IDOL	CL	CL-IDOL	NH_2CL	NH_2CL-IDOL
SW						
IDOL						
CL						
CL-IDOL						
NH_2CL						
NH_2CL-IDOL						

Abbreviations; SW=Akron source water, IDOL = iopamidol, CL=source water plus chlorine disinfection, CL-IDOL=source water plus Iopamidol plus chlorine disinfection, NH_2CL=source water plus chloramine disinfection, NH_2CL-IDOL=source water plus Iopamidol plus chloramine disinfection. In the pairwise comparison red indicates a significant difference between the paired groups, green indicates no significant difference between the paired groups.

Table 6.5. Experimental group 1: Comparative CHO cell acute genotoxicity of X-ray contrast agent Iopamidol in Akron source water with and without Iopamidol (IDOL) and disinfected with either chlorine (Cl) or monochloramine (NH_2Cl).

Sample	50%Tail DNA (CF) [a]	r^2 [b]	Lowest Genotoxic Conc. Factor [c]	ANOVA Test Statistic [d]
AOHTOX-SW	680.0	0.99	650	$F_{14, 39} = 12.0$; $P \leq 0.001$
AOHTOX-CL	223.3	0.96	175	$F_{9, 40} = 20.4$; $P \leq 0.001$
AOHTOX-IDOL	527.4	0.58	250	$F_{10, 43} = 3.35$; $P \leq 0.003$
AOHTOX-CL-IDOL	135.6	0.99	100	$F_{6, 21} = 32.8$; $P \leq 0.001$
AOHTOX-NH_2CL	545.4	0.99	350	$F_{14, 27} = 65.6$; $P \leq 0.001$
AOHTOX-NH_2CL-IDOL	279.4	0.98	250	$F_{12, 29} = 60.6$; $P \leq 0.001$

[a] The SCGE 50% Tail DNA value is the WSC sample concentration factor determined from a regression analysis of the data that was calculated to induce a 50% SCGE Tail DNA value. [b] r^2 is the coefficient of determination for the regression analysis upon which the SCGE %Tail DNA value was calculated. [c] The lowest genotoxic concentration was the lowest concentration factor of the WSC sample in the concentration-response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

Table 6.6. Experimental group 1: Test for significance among GTI values.

	SW	IDOL	CL	CL-IDOL	NH_2CL	NH_2CL-IDOL
SW						
IDOL						
CL						
CL-IDOL						
NH_2CL						
NH_2CL-IDOL						

Abbreviations; SW=Akron source water, IDOL = iopamidol, CL=source water plus chlorine disinfection, CL-IDOL=source water plus Iopamidol plus chlorine disinfection, NH_2CL=source water plus chloramine disinfection, NH_2CL-IDOL=source water plus Iopamidol plus chloramine disinfection. In the pairwise comparison red indicates a significant difference between the paired groups, green indicates no significant difference between the paired groups.

Table 6.7. Experimental group 2: Comparative CHO cell chronic cytotoxicity of X-ray contrast agents Iopromide, Iohexol, Diatrizoate and Iomeprol in Akron source water samples with and without Cl_2 disinfection.

Sample	LC_{50} (CF) [a]	r^2 [b]	Lowest Cytotoxic Conc. Factor [c]	ANOVA Test Statistic [d]
AOHTOX Iopromide	53.4	0.92	25.0	$F_{10, 117} = 39.7$; $P \leq 0.001$
AOHTOX Iopromide + Cl_2	38.0	0.93	15.0	$F_{11, 172} = 22.9$; $P \leq 0.001$
AOHTOX Iohexol	46.6	0.96	20.0	$F_{16, 167} = 16.4$; $P \leq 0.001$
AOHTOX Iohexol + Cl_2	14.4	0.93	7.5	$F_{11, 124} = 26.1$; $P \leq 0.001$
AOHTOX Diatrizoate	74.0	0.87	20.0	$F_{15, 168} = 24.9$; $P \leq 0.001$
AOHTOX Diatrizoate + Cl_2	26.9	0.96	15.0	$F_{11, 124} = 37.7$; $P \leq 0.001$
AOHTOX Iomeprol	44.0	0.96	10.0	$F_{10, 85} = 26.7$; $P \leq 0.001$
AOHTOX Iomeprol + Cl_2	22.9	0.99	7.5	$F_{15, 164} = 31.9$; $P \leq 0.001$

[a] The LC50 value is the fold concentration factor of the WCS sample, determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. [b] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated. [c] Lowest cytotoxic concentration was the lowest concentration factor of the WSC in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls. [d] The degrees of freedom for the between-groups and residual associated with the calculated F-test result and the resulting probability value.

Table 6.8. Experimental group 2: Test for significance among CTI values.

	SW	CL	IPRO	IPRO CL	IHX	IHX CL	DTZ	DTZ CL	IOME	IOME CL
SW										
CL										
IPRO										
IPROCL										
IHX										
IHXCL										
DTZ										
DTZCL										
IOME										
IOMECL										

Abbreviations; SW=Akron source water, CL=Akron source water plus Cl_2, IPRO=Iopromide, IPROCL=Iopromide plus Cl_2, IHX=Iohexol, IHXCL=Iohexol plus Cl_2, DTZ=Diatrizoate, DTZCL=Diatrizoate + Cl_2, IOME=Iomeprol, IOMECL=Iomeprol + Cl_2. In the pairwise comparison red indicates a significant difference between the paired groups, green indicates no significant difference between the paired groups.

Table 6.9. Experimental group 2: Comparative CHO cell acute genotoxicity of X-ray contrast agents Iopromide, Iohexol, Diatrizoate and Iomeprol in Akron source water samples with and without Cl_2 disinfection.

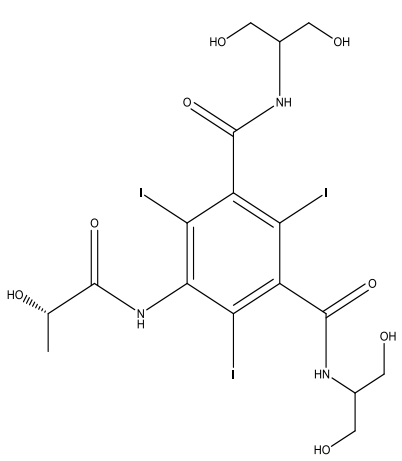
Sample	50%Tail DNA (CF) [a]	r^2 [b]	Lowest Genotoxic Conc. Factor [c]	ANOVA Test Statistic [d]
Iopromide	508.6	0.99	460.0	$F_{9, 34} = 19.7$; $P \leq 0.001$
Iopromide + Cl_2	267.0	0.96	250.0	$F_{11, 38} = 44.6$; $P \leq 0.001$
Iohexol	562.3	0.95	480.0	$F_{14, 39} = 7.79$; $P \leq 0.001$
Iohexol + Cl_2	169.0	0.98	140.0	$F_{8, 40} = 28.9$; $P \leq 0.001$
Diatrizoate	789.5	0.99	700.0	$F_{13, 58} = 30.9$; $P \leq 0.001$
Diatrizoate + Cl_2	206.7	0.99	140.0	$F_{12, 49} = 193.8$; $P \leq 0.001$
Iomeprol	706.3	0.95	700.0	$F_{12, 31} = 7.39$; $P \leq 0.001$
Iomeprol + Cl_2	211.2	0.89	150.0	$F_{9, 32} = 18.8$; $P \leq 0.001$

[a] The SCGE 50% Tail DNA value is the WSC sample concentration factor determined from a regression analysis of the data that was calculated to induce a 50% SCGE Tail DNA value. [b] r^2 is the coefficient of determination for the regression analysis upon which the SCGE %Tail DNA value was calculated. [c] The lowest genotoxic concentration was the lowest concentration factor of the WSC sample in the concentration−response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

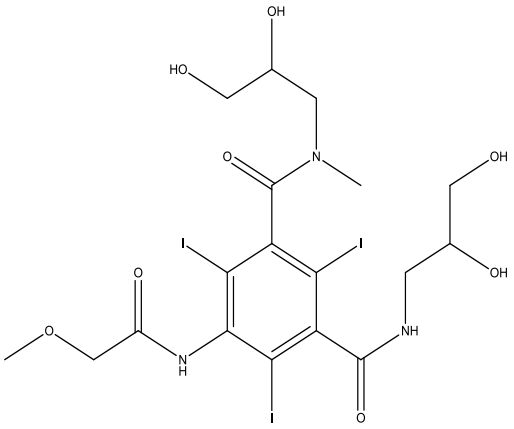
Table 6.10. Experimental group 2: Test for significance among GTI values.

	SW	CL	IPRO	IPRO CL	IHX	IHX CL	DTZ	DTZ CL	IOME	IOME CL
SW										
CL										
IPRO										
IPROCL										
IHX										
IHXCL										
DTZ										
DTZCL										
IOME										
IOMECL										

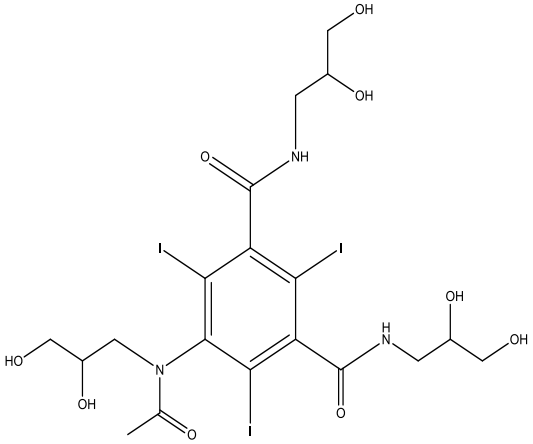
Abbreviations; SW=Akron source water, CL=Akron source water plus Cl_2, IPRO=Iopromide, IPROCL=Iopromide plus Cl_2, IHX=Iohexol, IHXCL=Iohexol plus Cl_2, DTZ=Diatrizoate, DTZCL=Diatrizoate + Cl_2, IOME=Iomeprol, IOMECL=Iomeprol + Cl_2. In the pairwise comparison red indicates a significant difference between the paired groups, green indicates no significant difference between the paired groups.



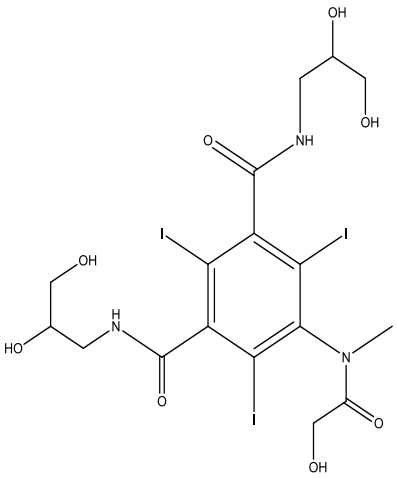
Iopamidol
CAS No. 62883-00-5
776.85 Da



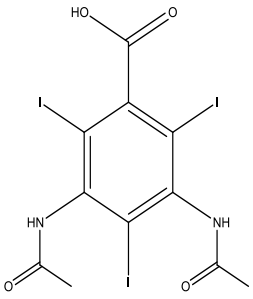
Iopromide
CAS No. 73334-07-3
790.87 Da



Iohexol
CAS No. 66108-95-0
820.88 Da



Iomeprol
CAS No. 78649-41-9
776.85 Da



Diatrizoate
CAS No. 737-31-5
613.77 Da

Figure 6.1. Chemical structures of iodinated X-ray contrast agents (ICM) analyzed in this study.

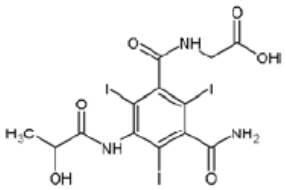
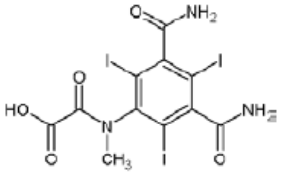
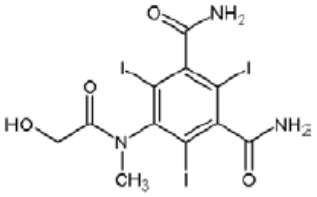
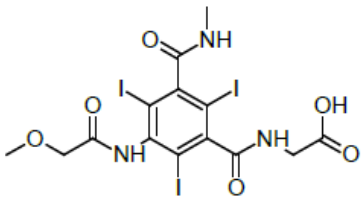
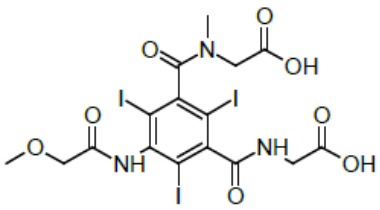
Chemical Structure Name	Chemical Structure Name	Chemical Structure Name
		
Iopamidol TP687	Iomeprol TP643	Iomeprol TP629
		
Iopromide TP701A	Iopromide TP759	

Figure 6.2. Chemical structures of some transformation products of X-ray contrast media.

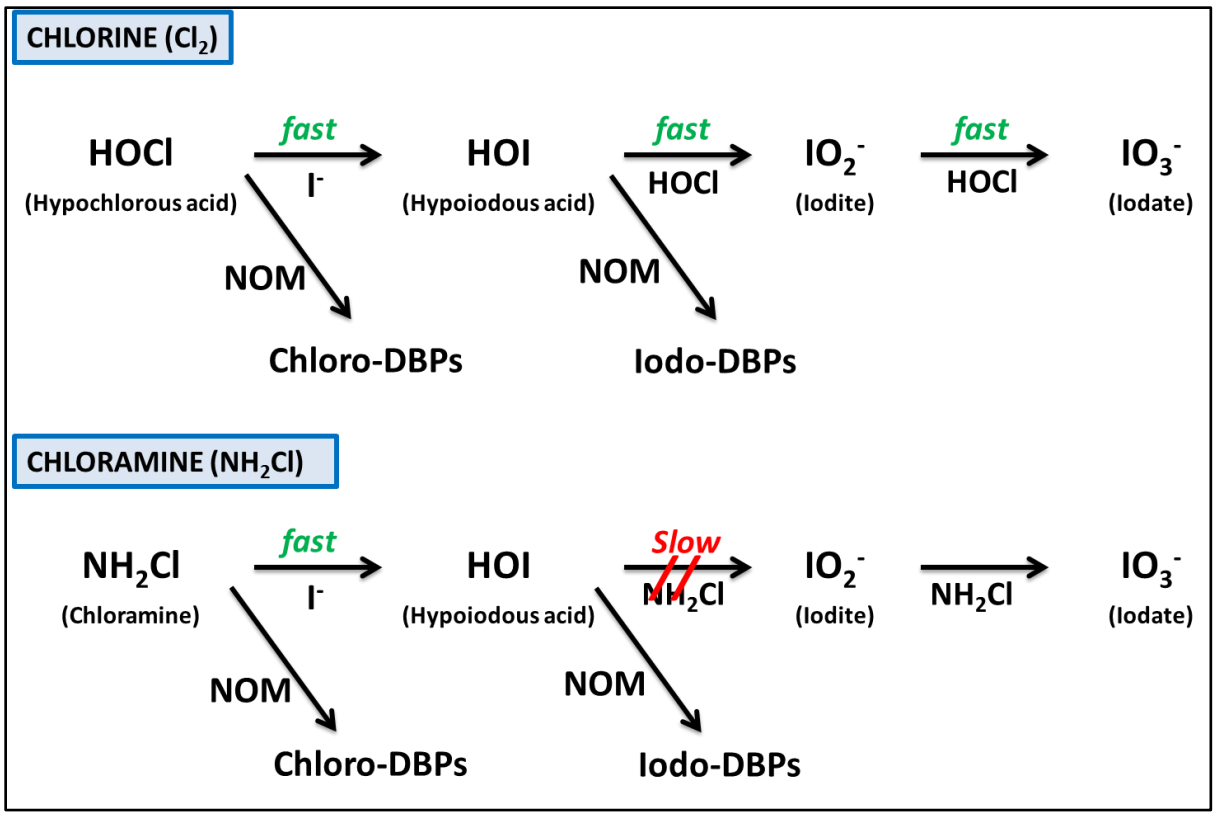


Figure 6.3. Chemical mechanisms involved in the formation of iodo-DBPs by chlorine and chloramine. Figure based on data by Bichsel and von Guten [14].

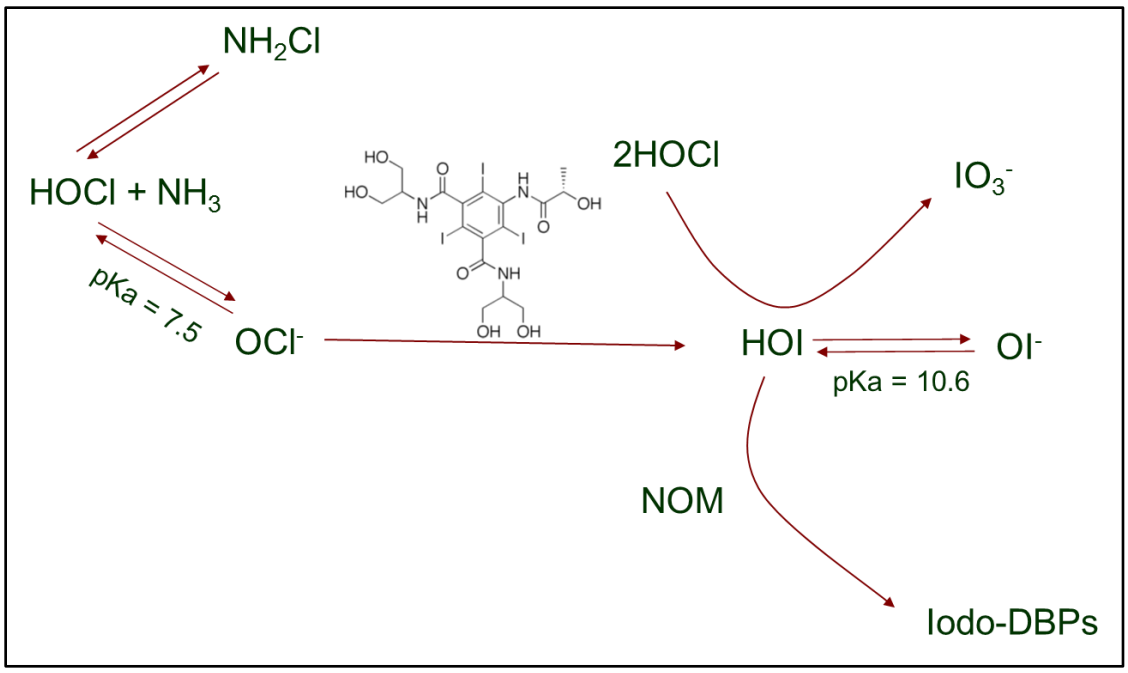


Figure 6.4. Chemical mechanism involved in the formation of iodo-DBPs by Iopamidol [6].

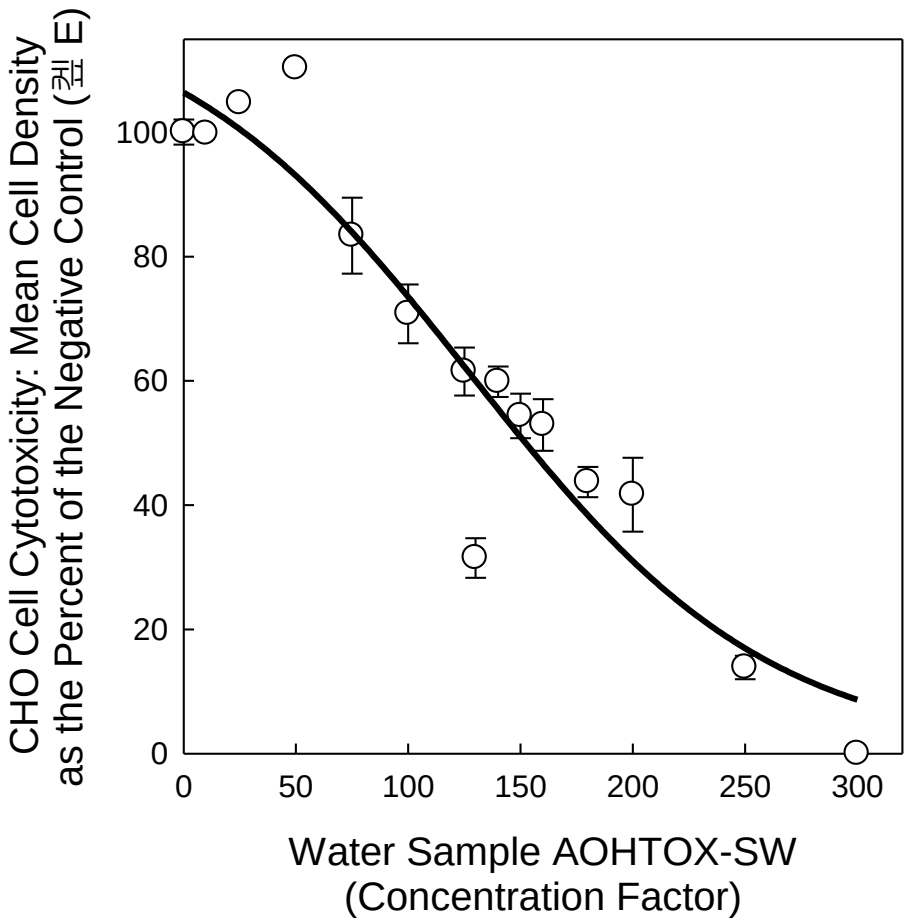


Figure 6.5. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-SW.

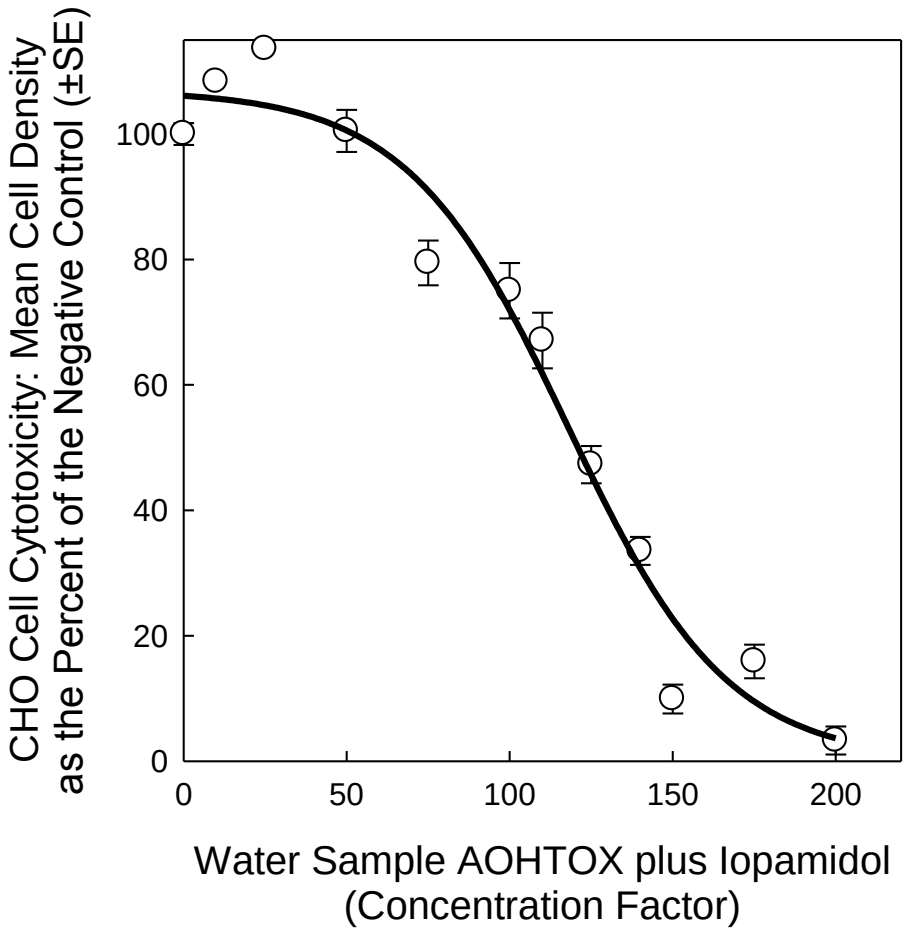


Figure 6.6. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-IDOL.

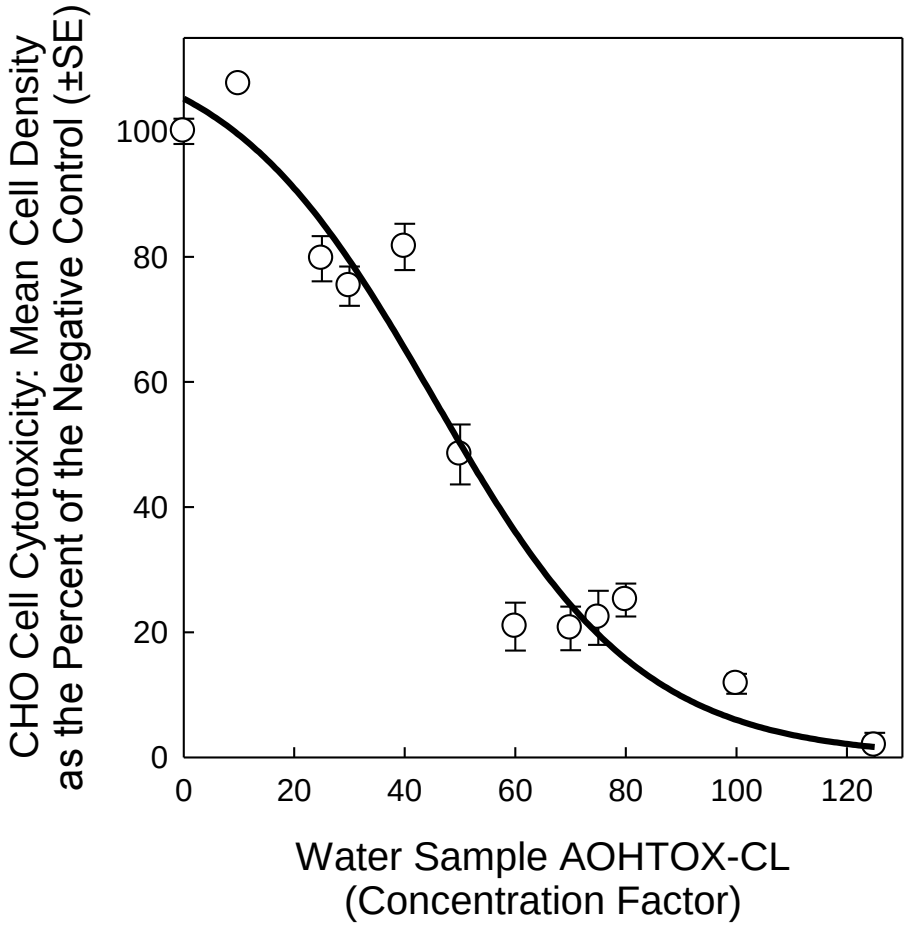


Figure 6.7. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL.

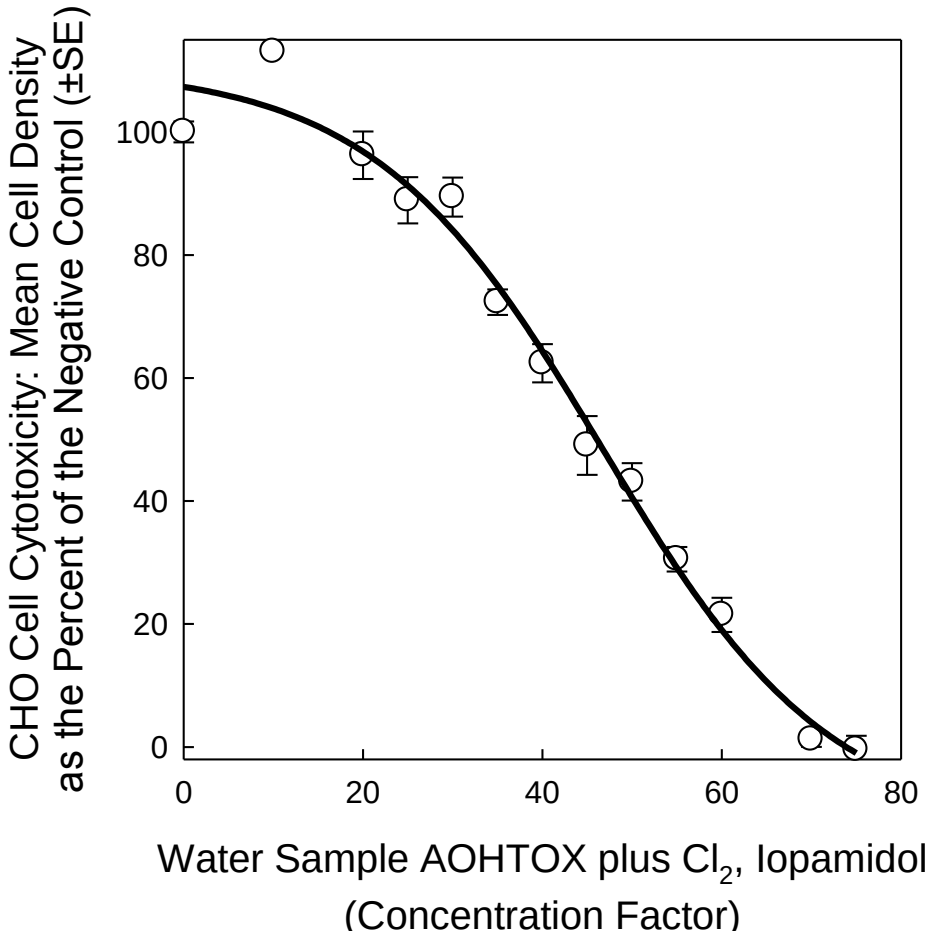


Figure 6.8. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL-IDOL.

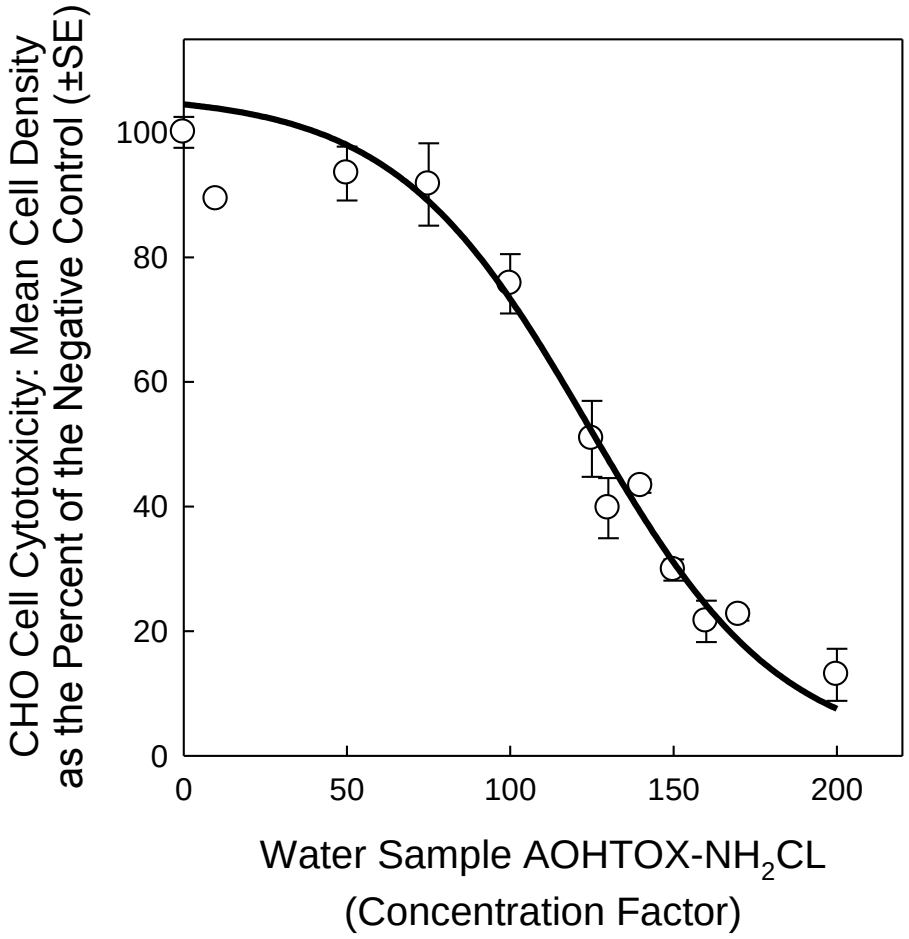


Figure 6.9. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-NH_2CL.

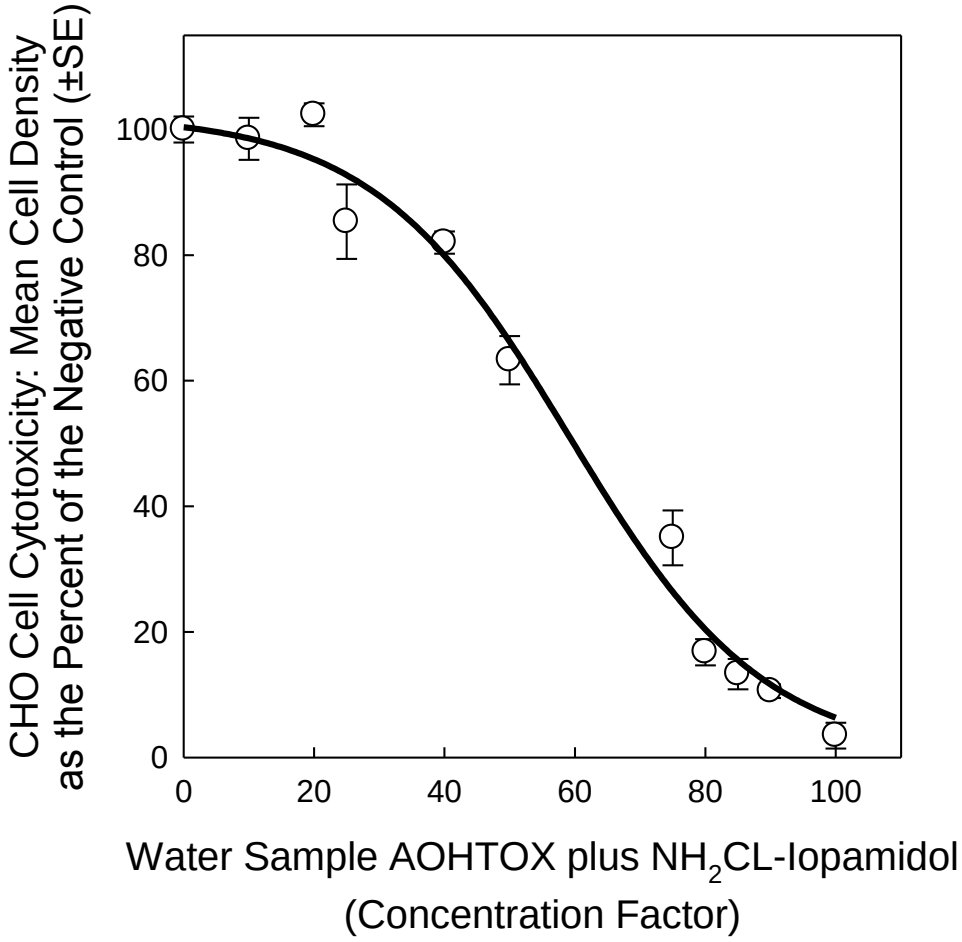


Figure 6.10. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-NH_2CL-IDOL.

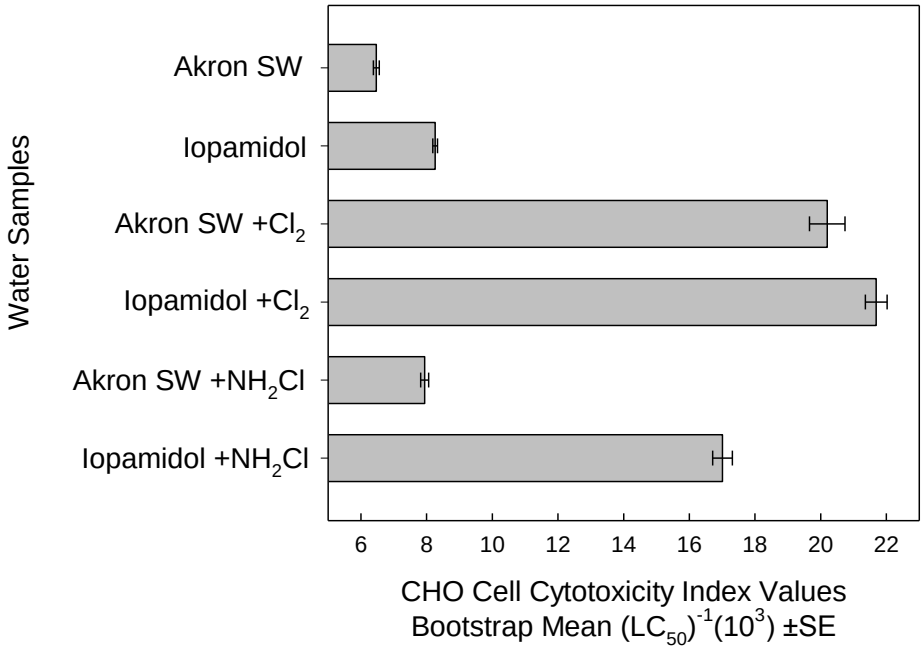


Figure 6.11. Experimental group 1: CHO cell chronic cytotoxicity index (CTI) values.

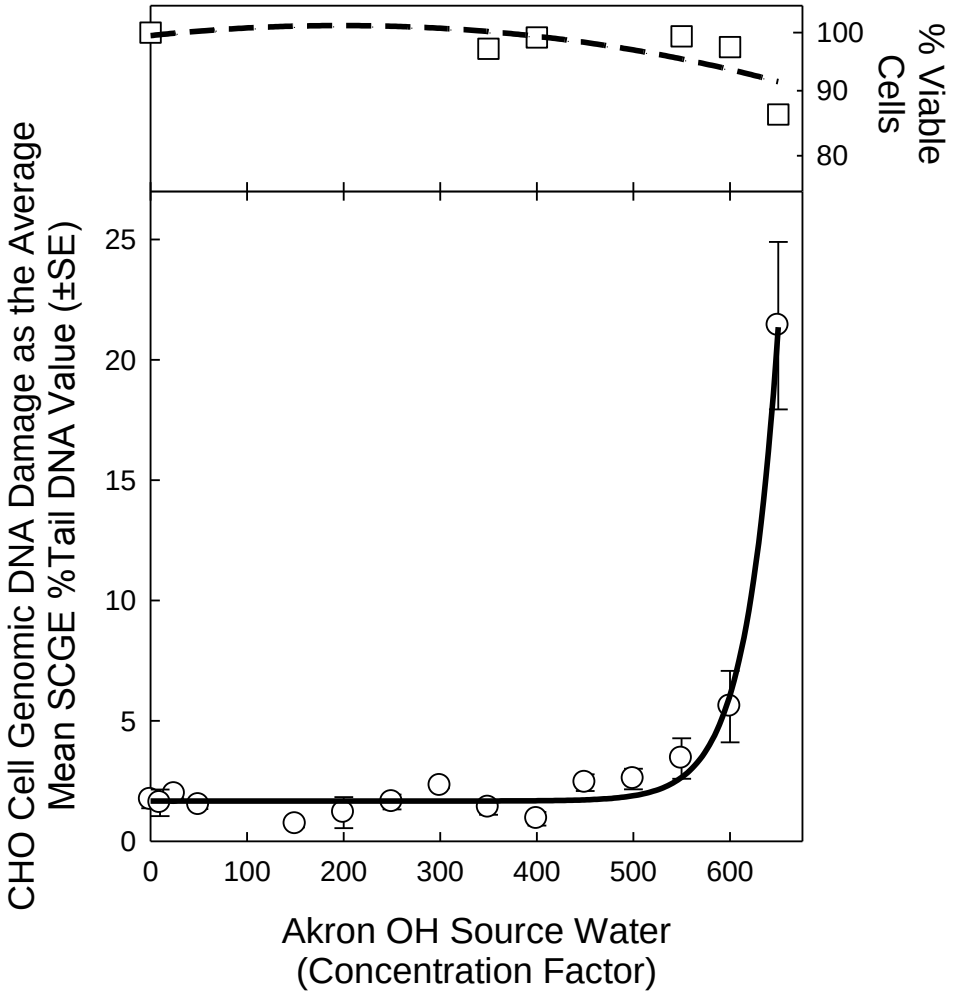


Figure 6.12. CHO cell acute genotoxicity concentration-response curve of AOHTOX-SW.

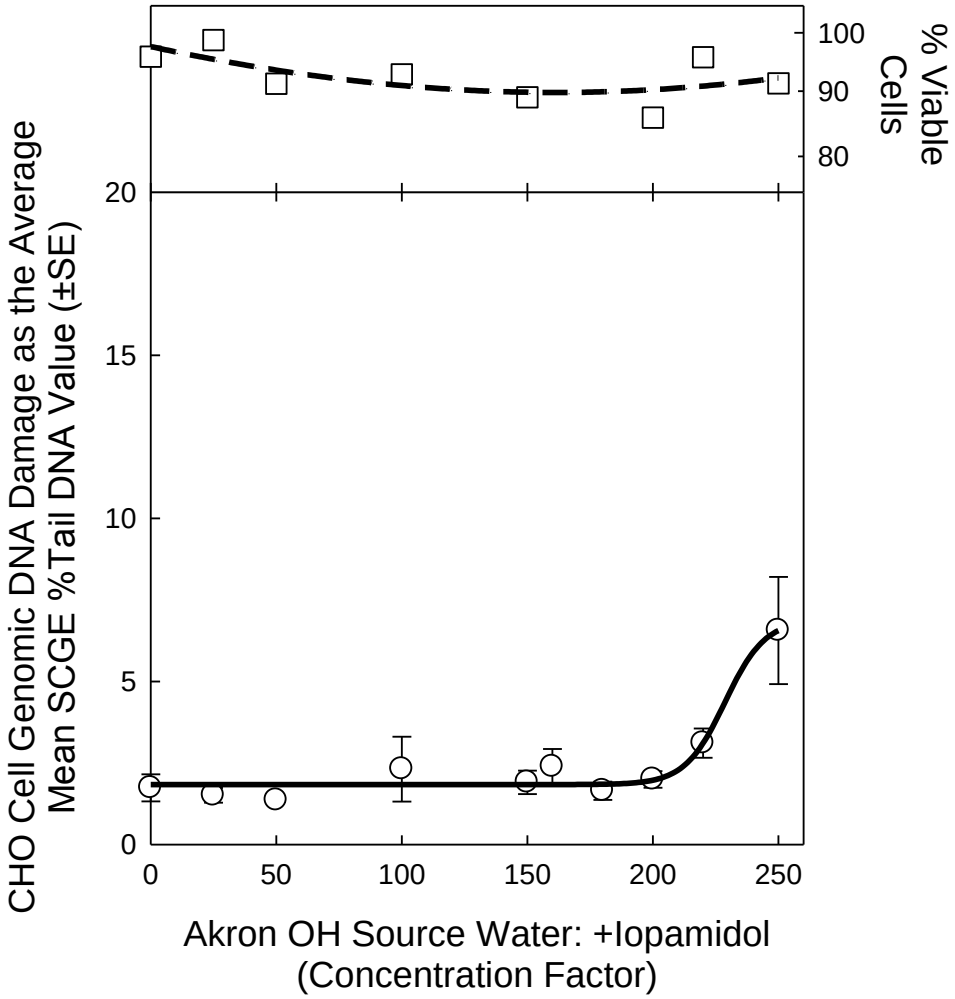


Figure 6.13. CHO cell acute genotoxicity concentration-response curve of AOHTOX-IODOL.

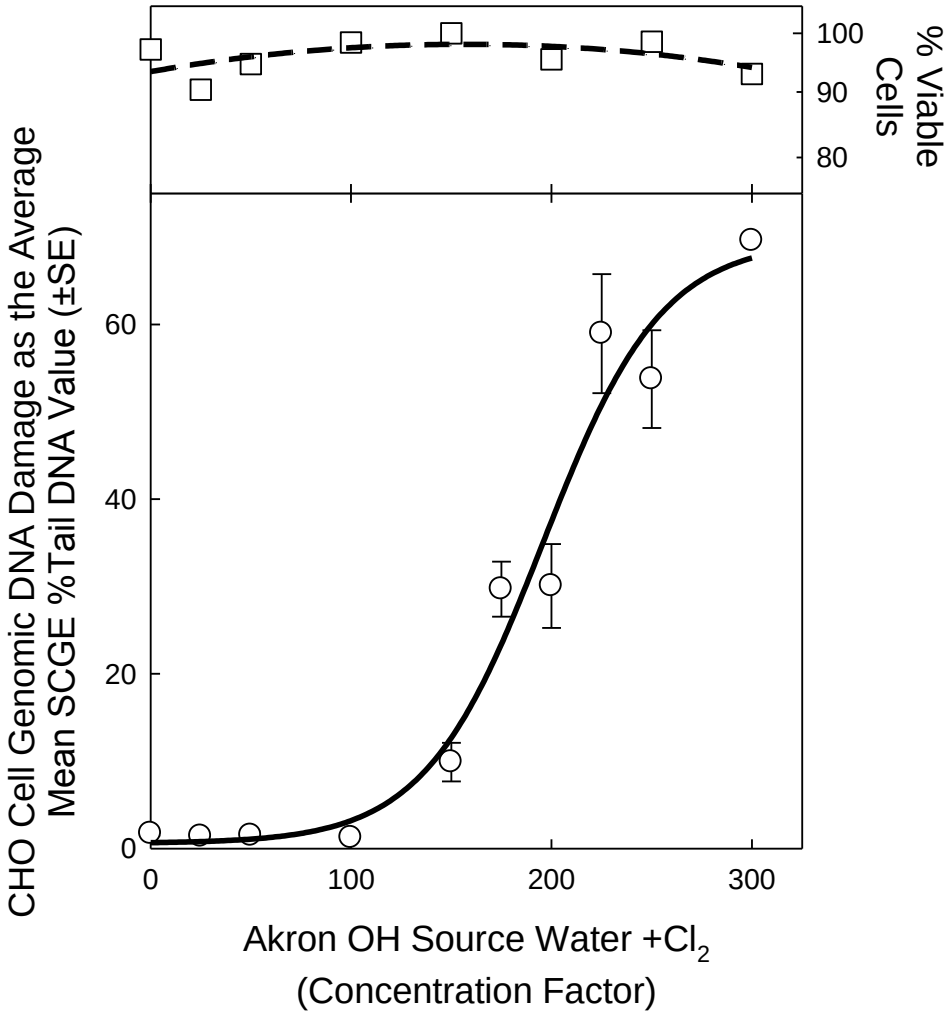


Figure 6.14. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL.

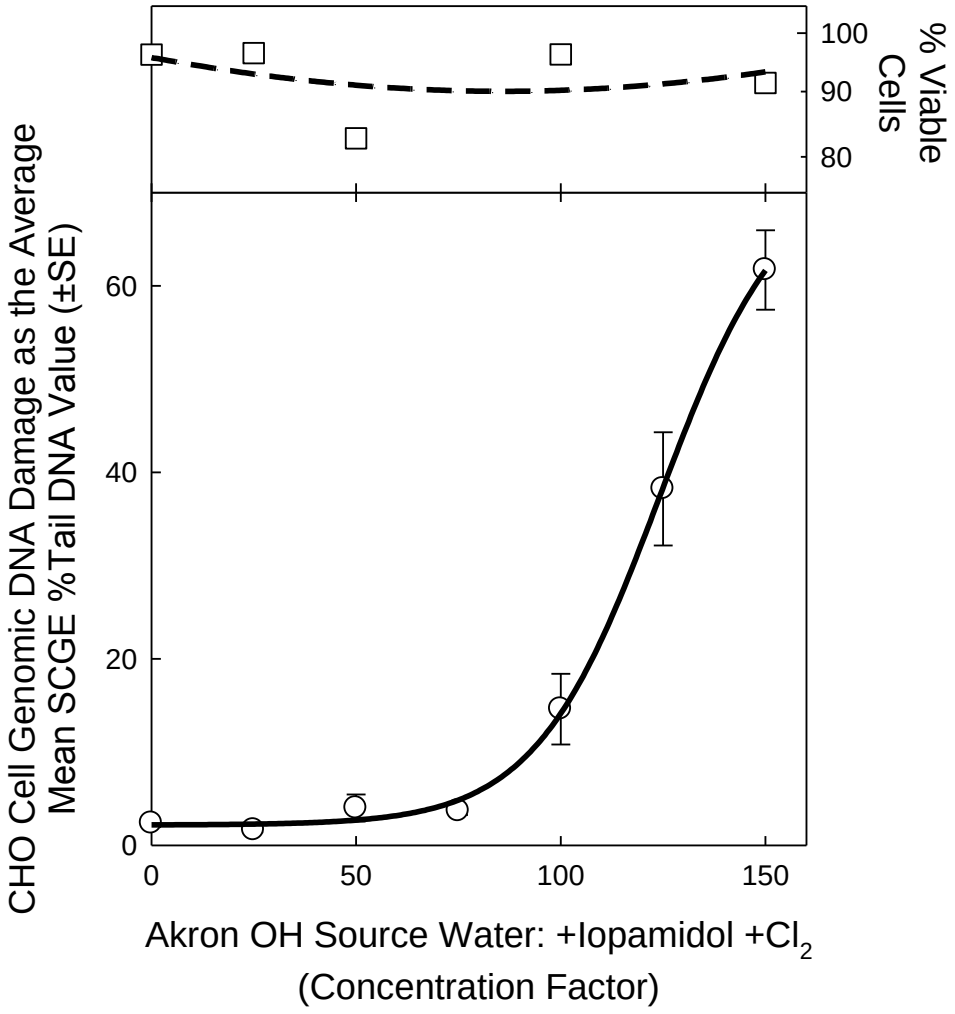


Figure 6.15. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL-IODOL.

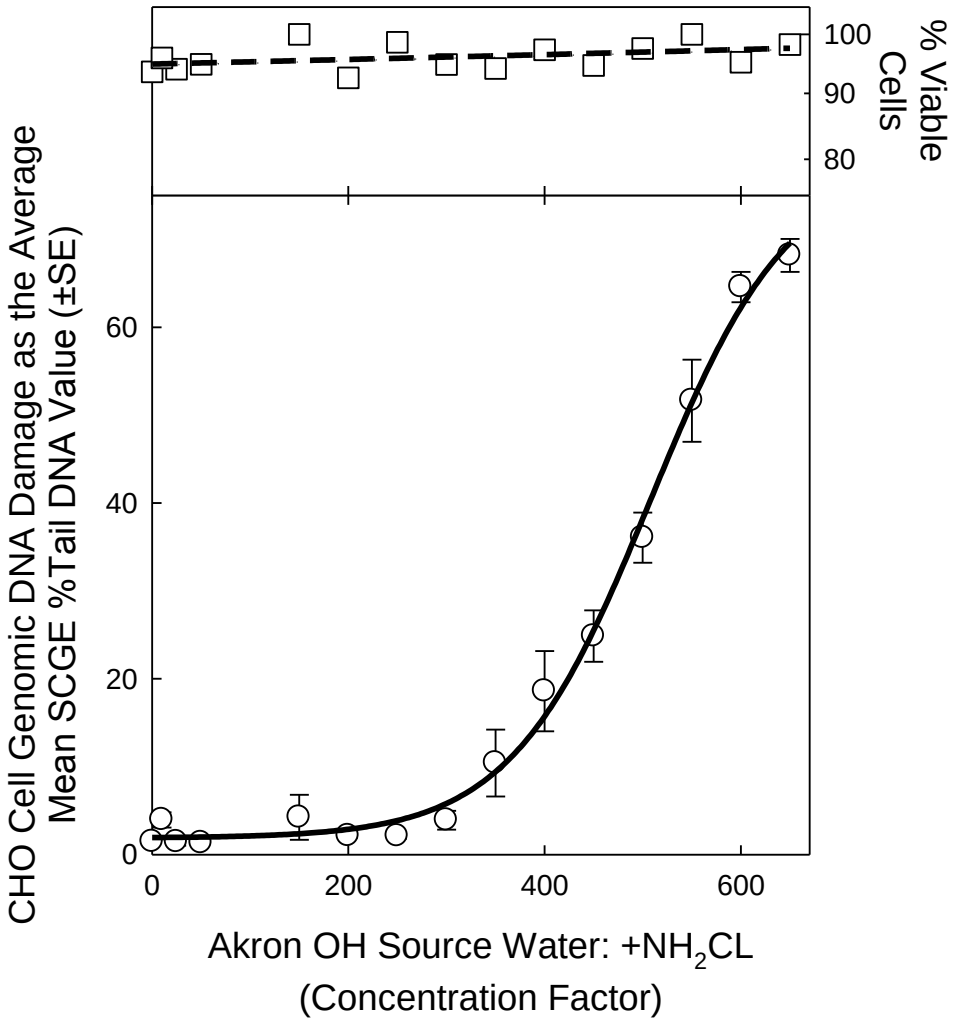


Figure 6.16. CHO cell acute genotoxicity concentration-response curve of AOHTOX-NH_2CL.

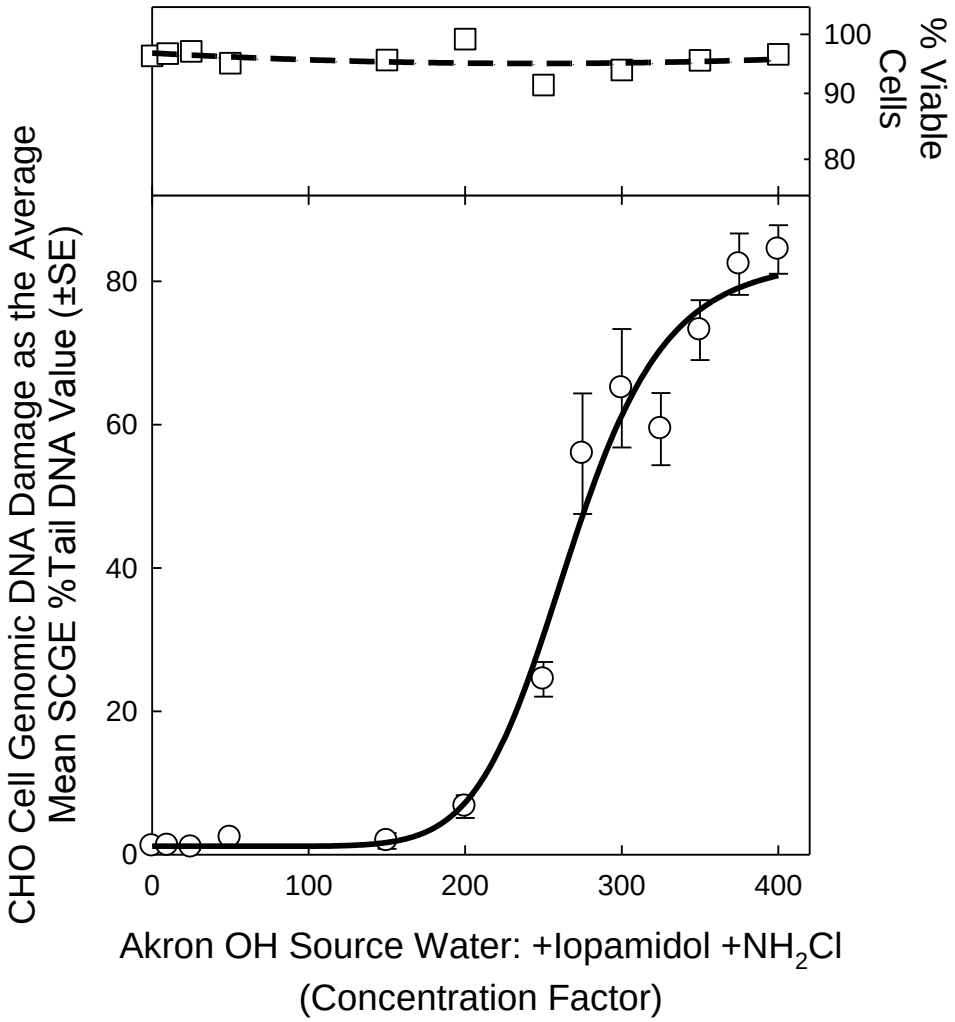


Figure 6.17. CHO cell acute genotoxicity concentration-response curve of AOHTOX-NH_2CL-IODOL.

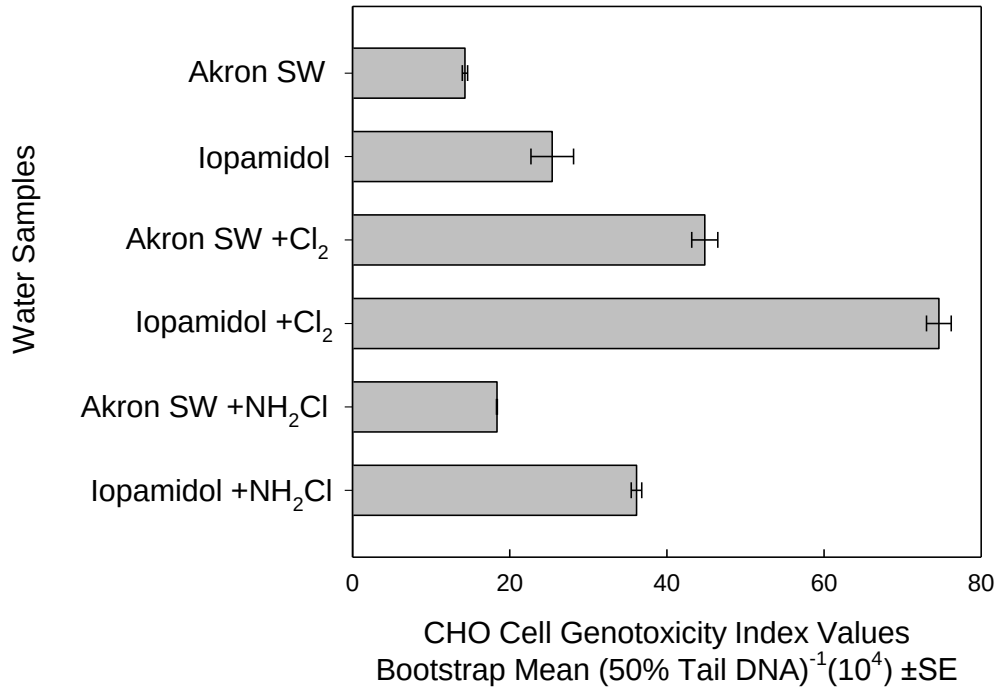


Figure 6.18. Experimental group 1: CHO cell acute genotoxicity index (GTI) values.

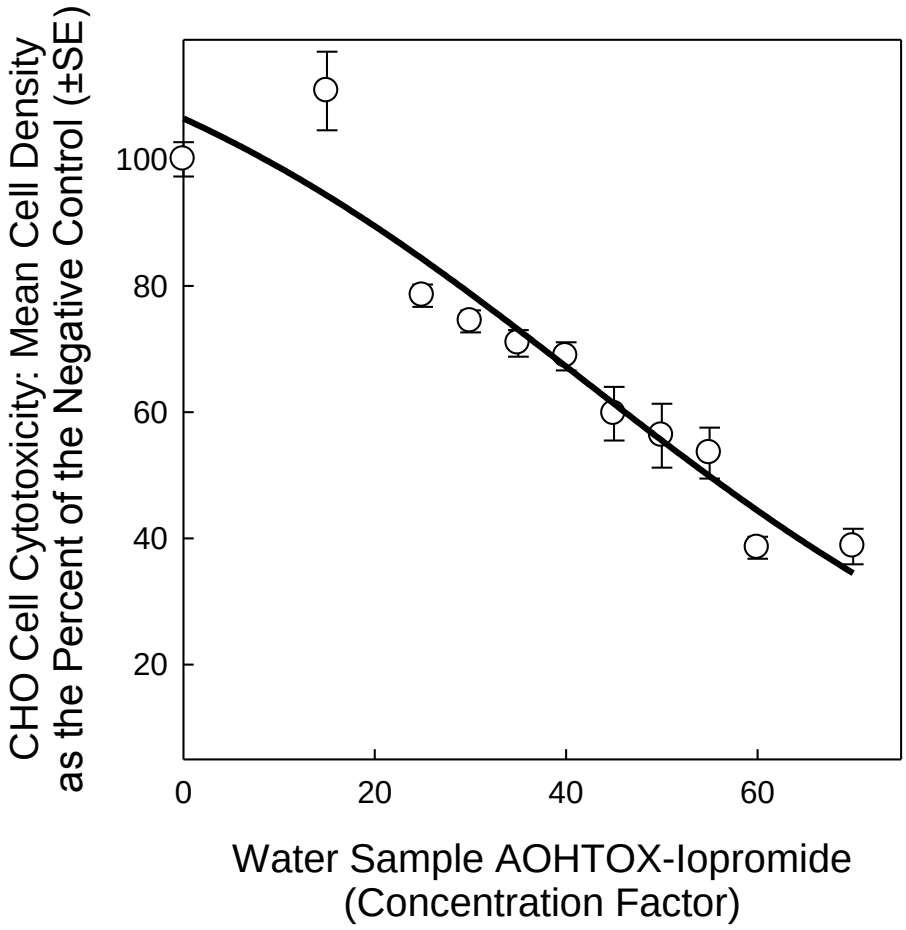


Figure 6.19. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-Iopromide.

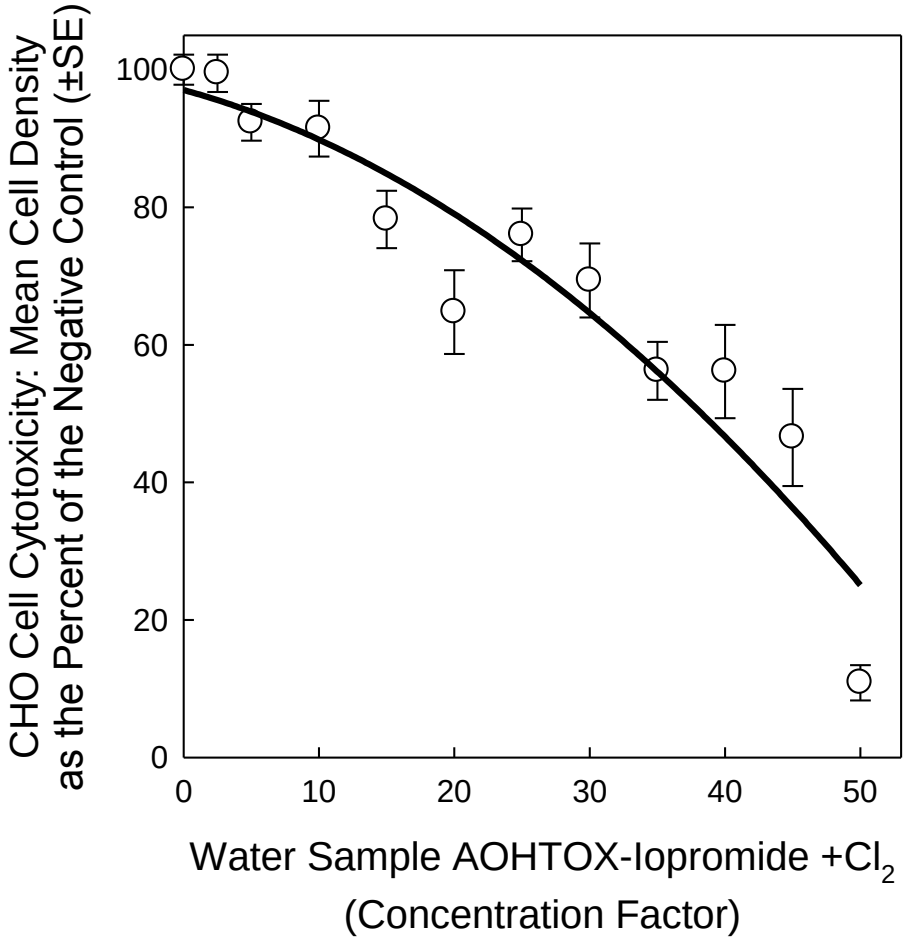


Figure 6.20. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL-Iopromide.

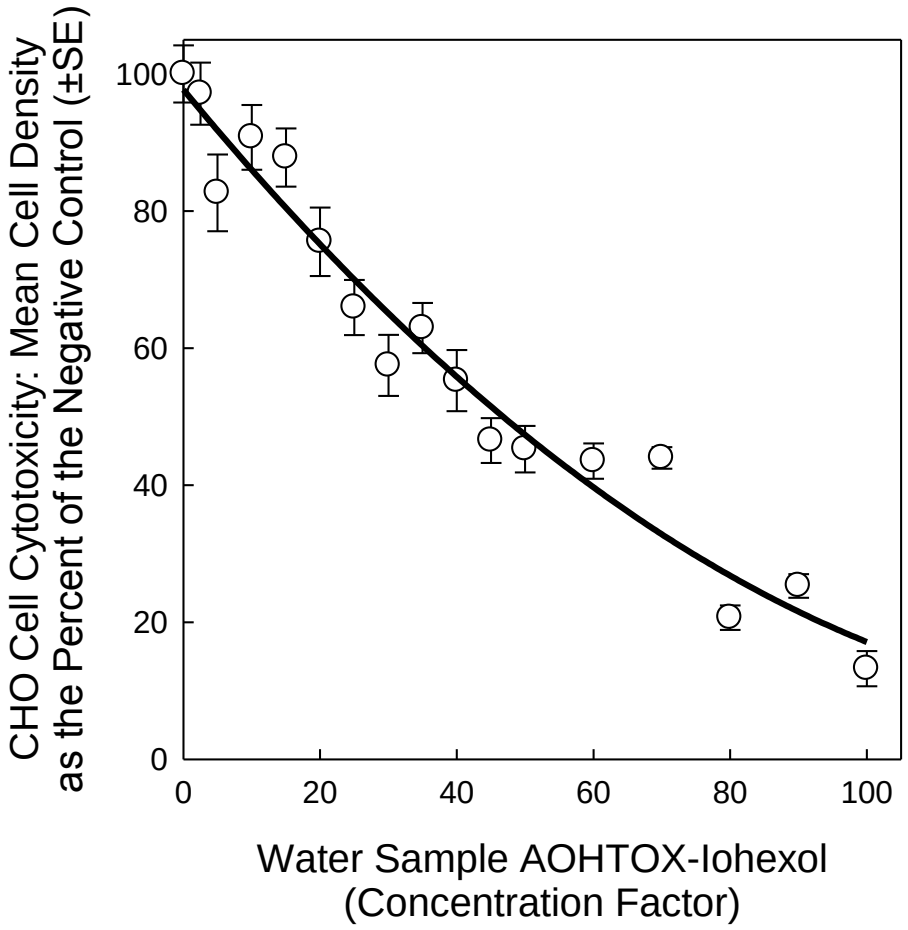


Figure 6.21. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-Iohexol.

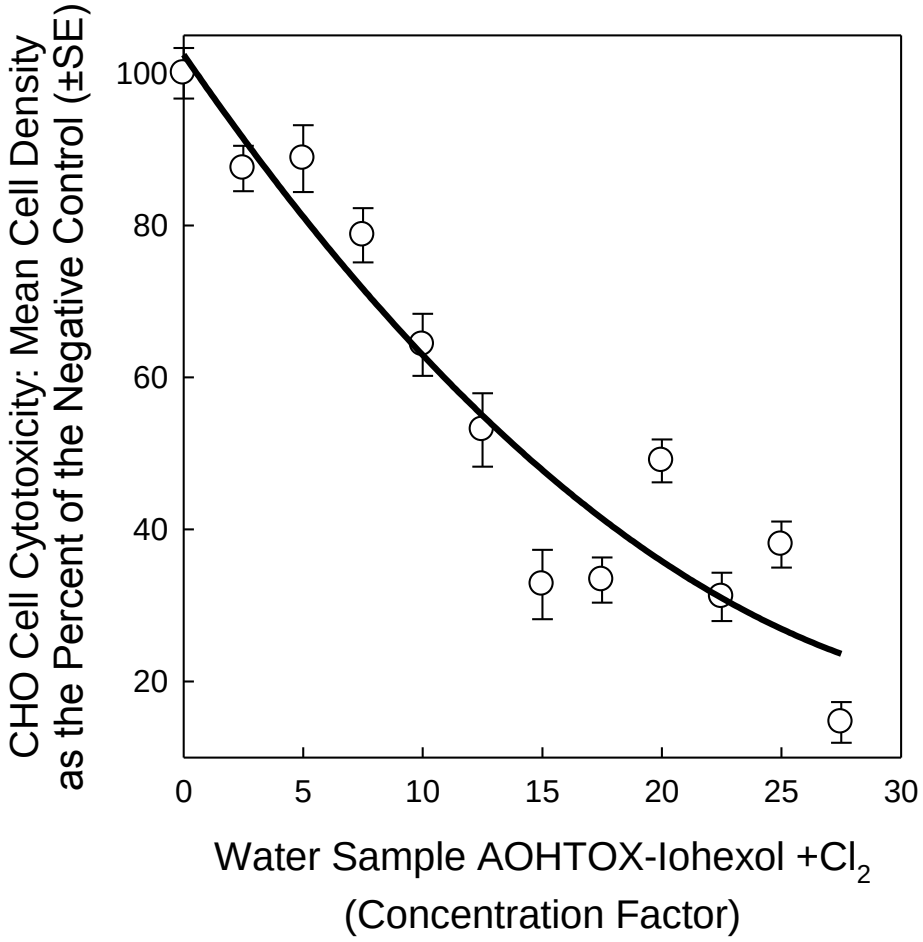


Figure 6.22. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL-Iohexol.

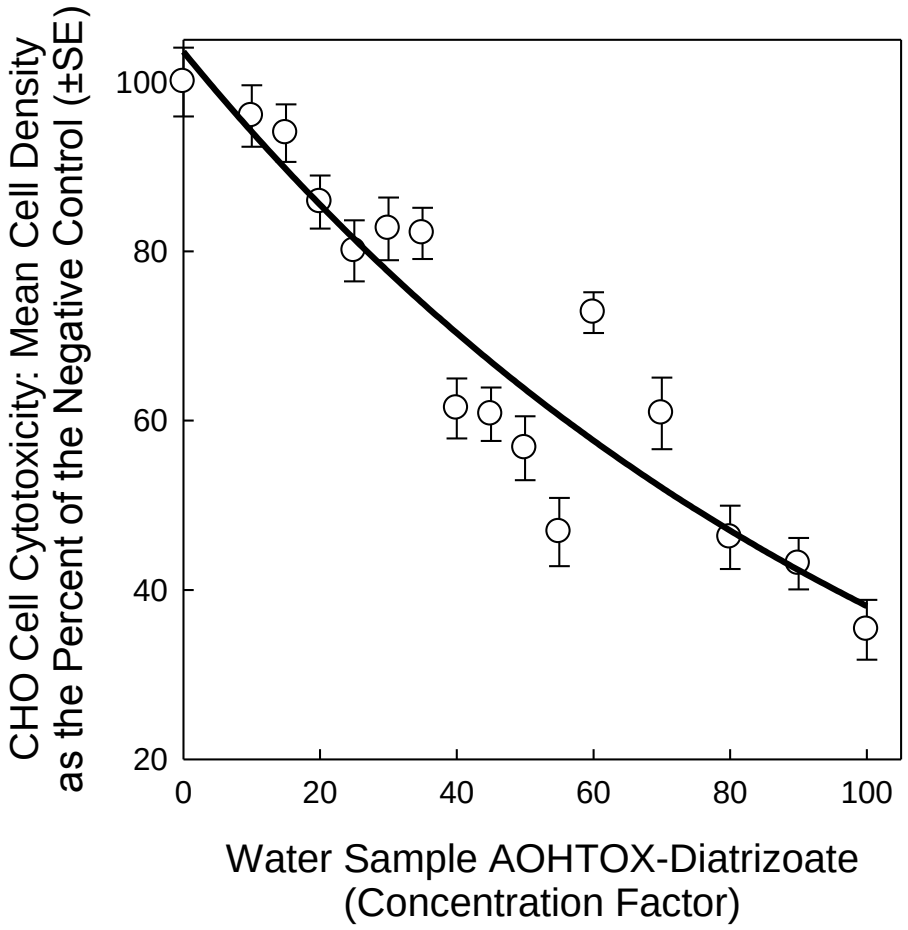


Figure 6.23. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-Diatrizoate.

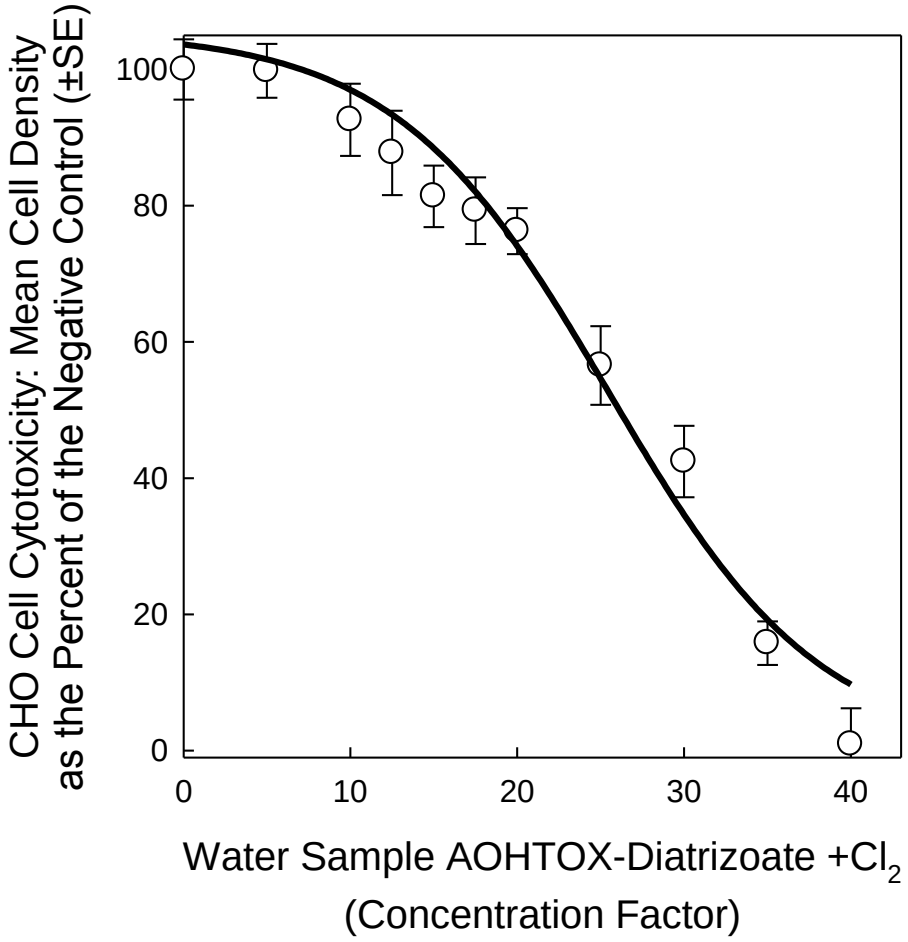


Figure 6.24. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL-Diatrizoate.

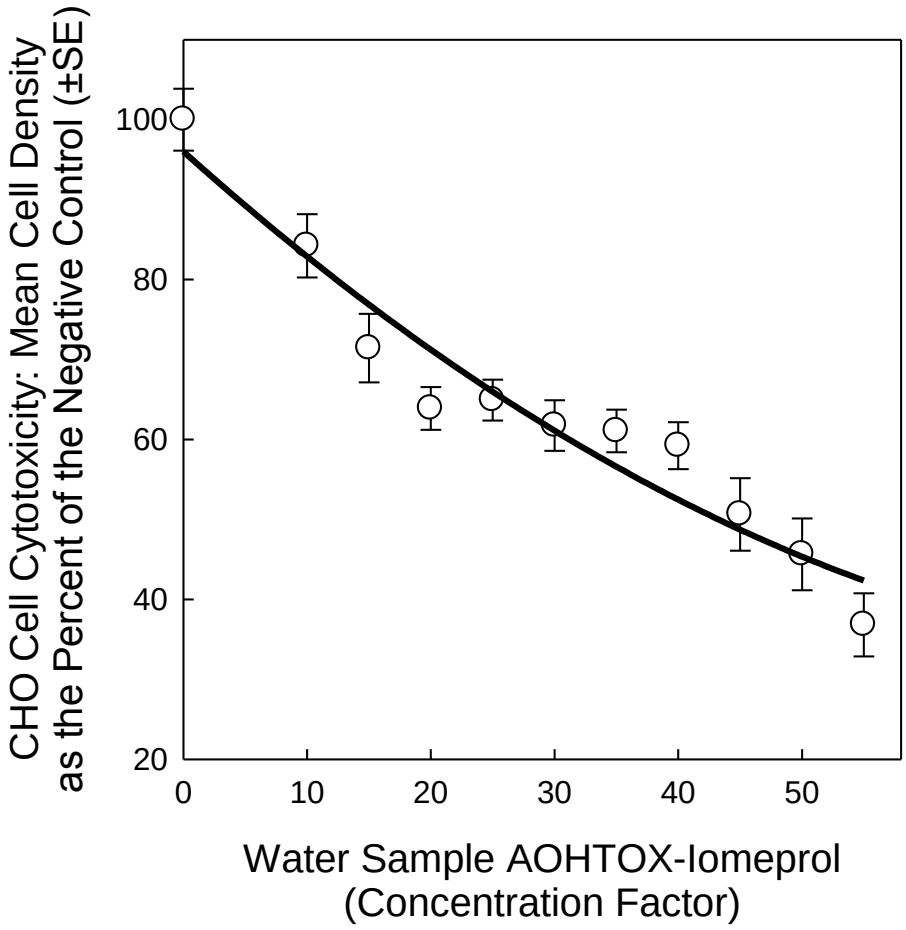


Figure 6.25. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-Iomeprol.

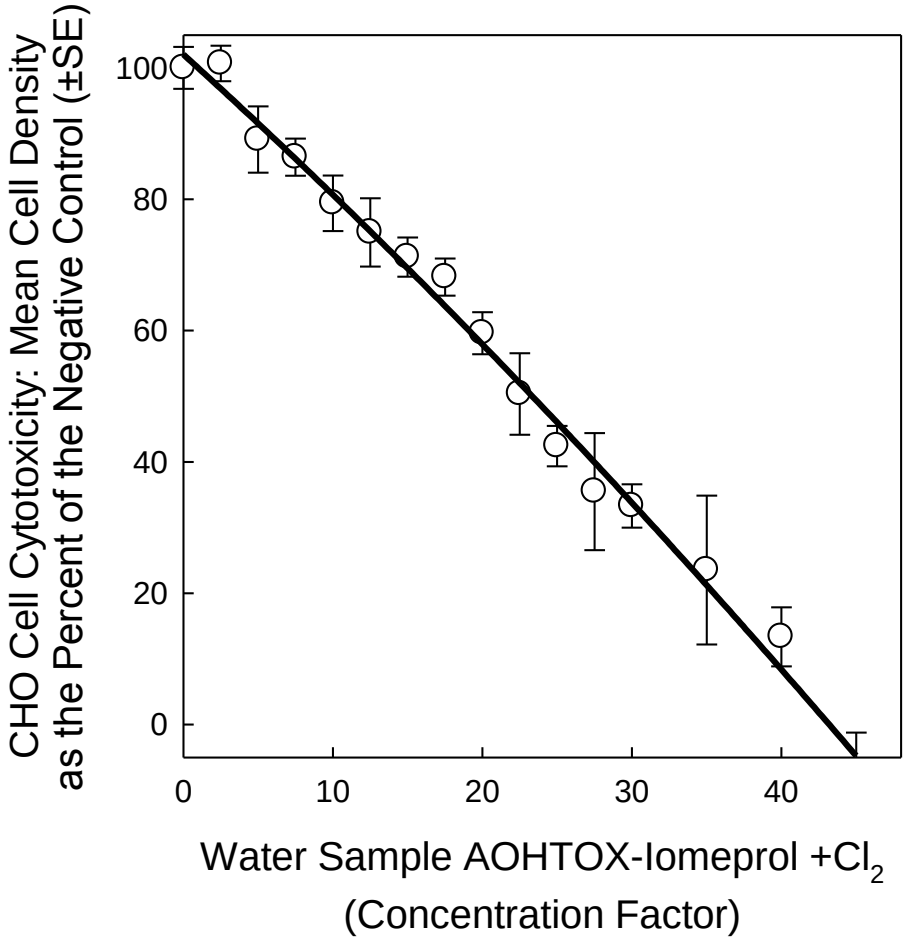


Figure 6.26. CHO cell chronic cytotoxicity concentration-response curve of AOHTOX-CL-Iomeprol.

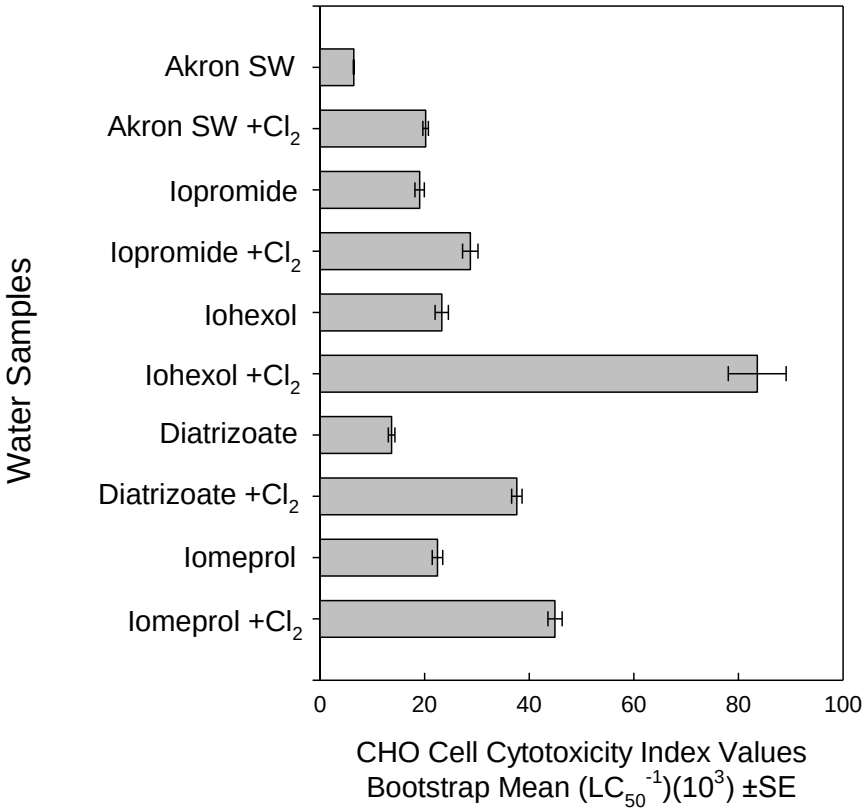


Figure 6.27. Experimental group 2: CHO cell chronic cytotoxicity index (CTI) values.

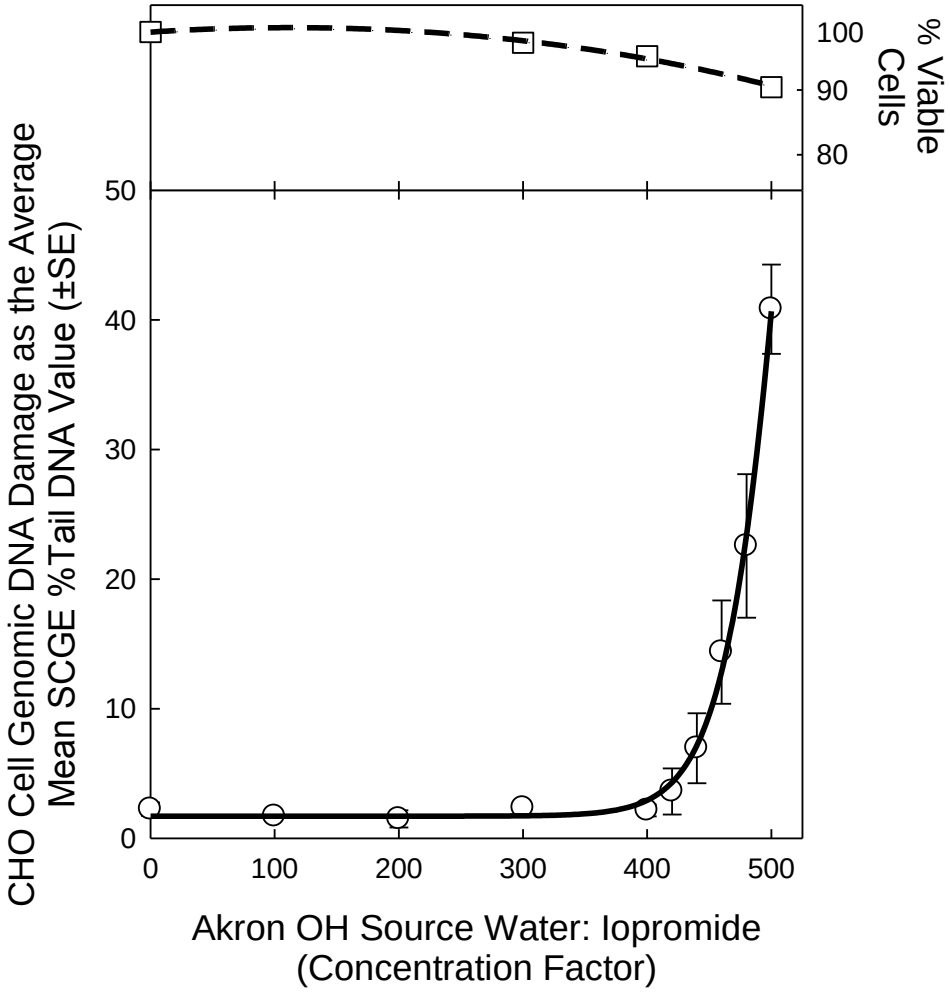


Figure 6.28. CHO cell acute genotoxicity concentration-response curve of AOHTOX-Iopromide.

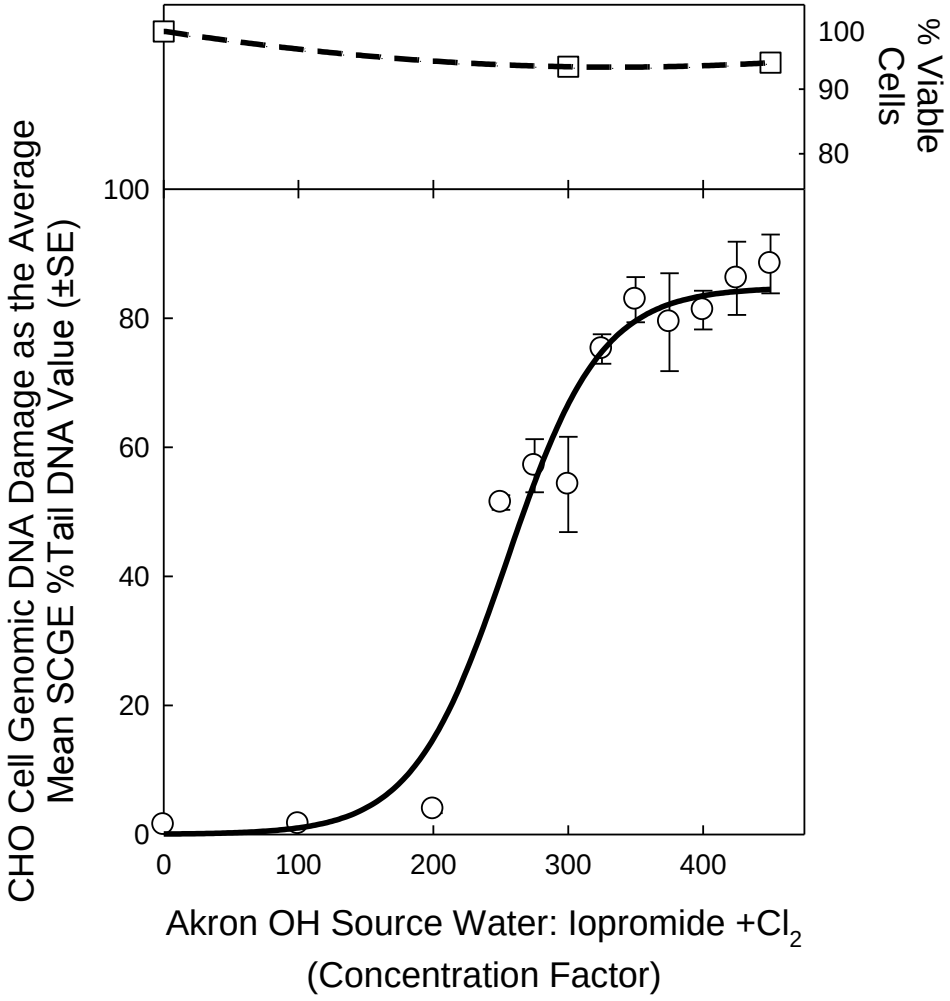


Figure 6.29. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL-Iopromide.

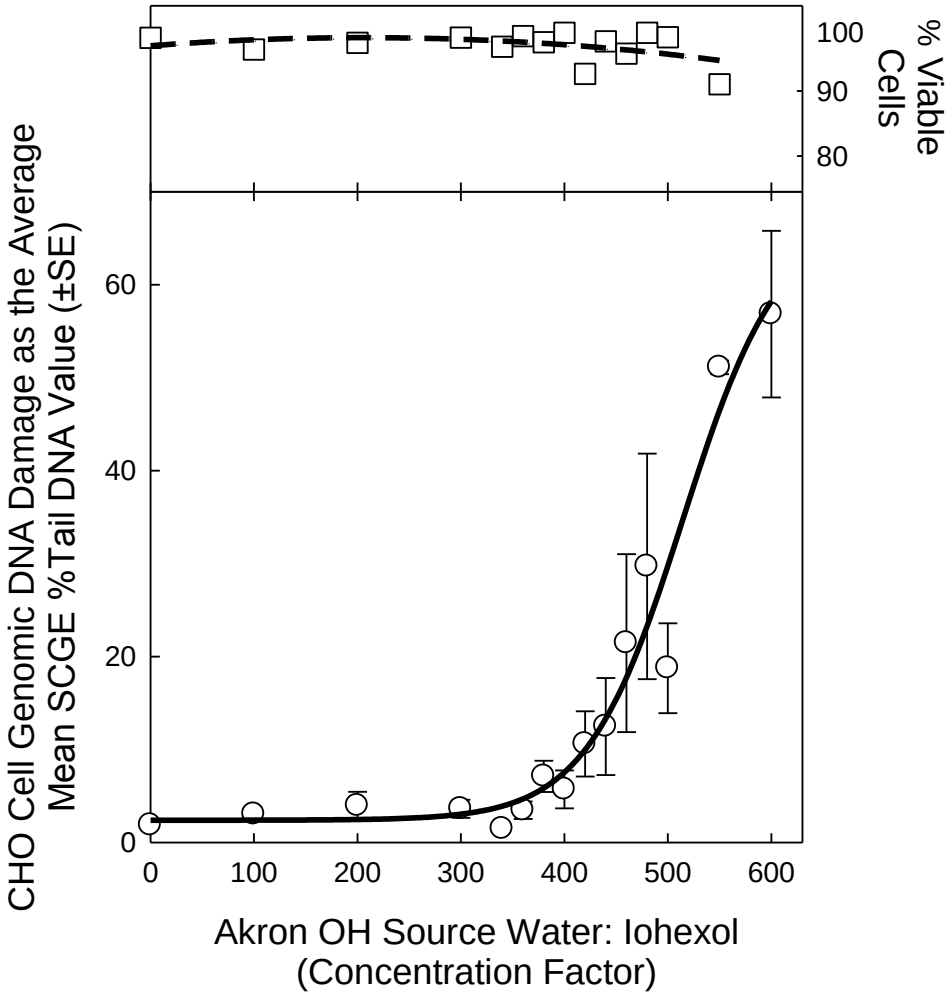


Figure 6.30. CHO cell acute genotoxicity concentration-response curve of AOHTOX-Iohexol.

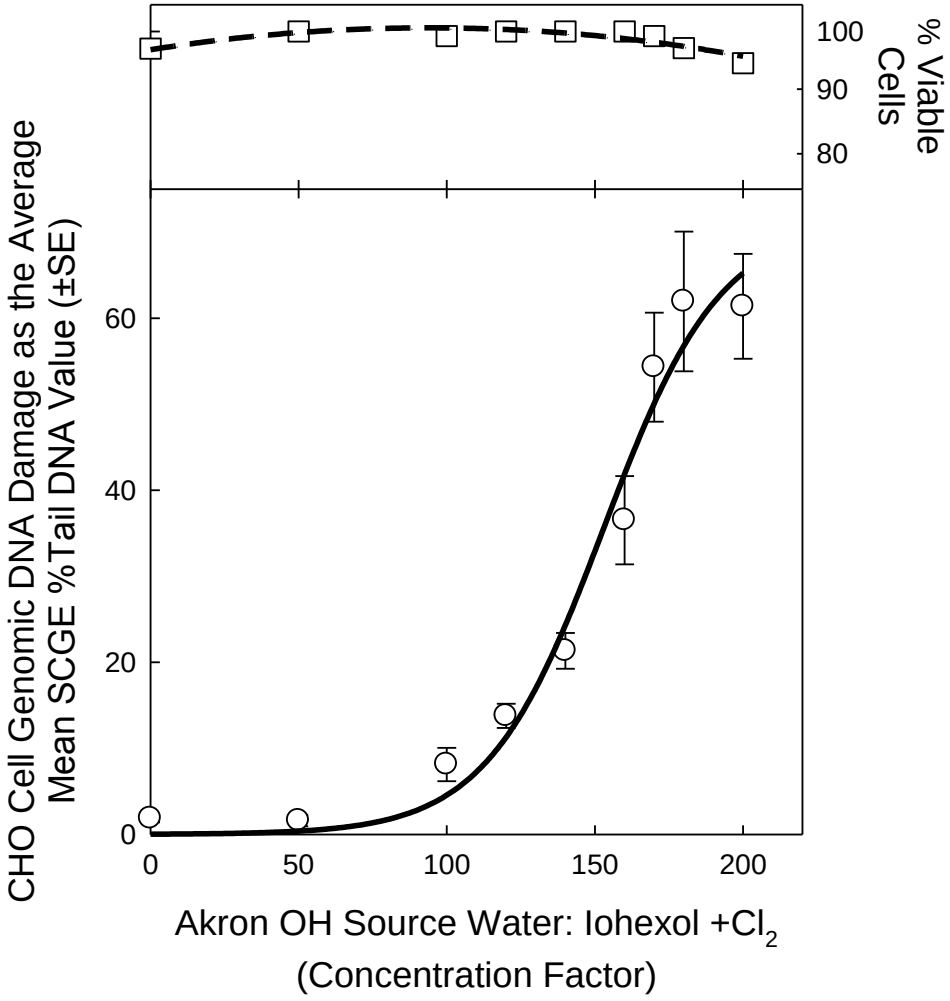


Figure 6.31. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL-Iohexol.

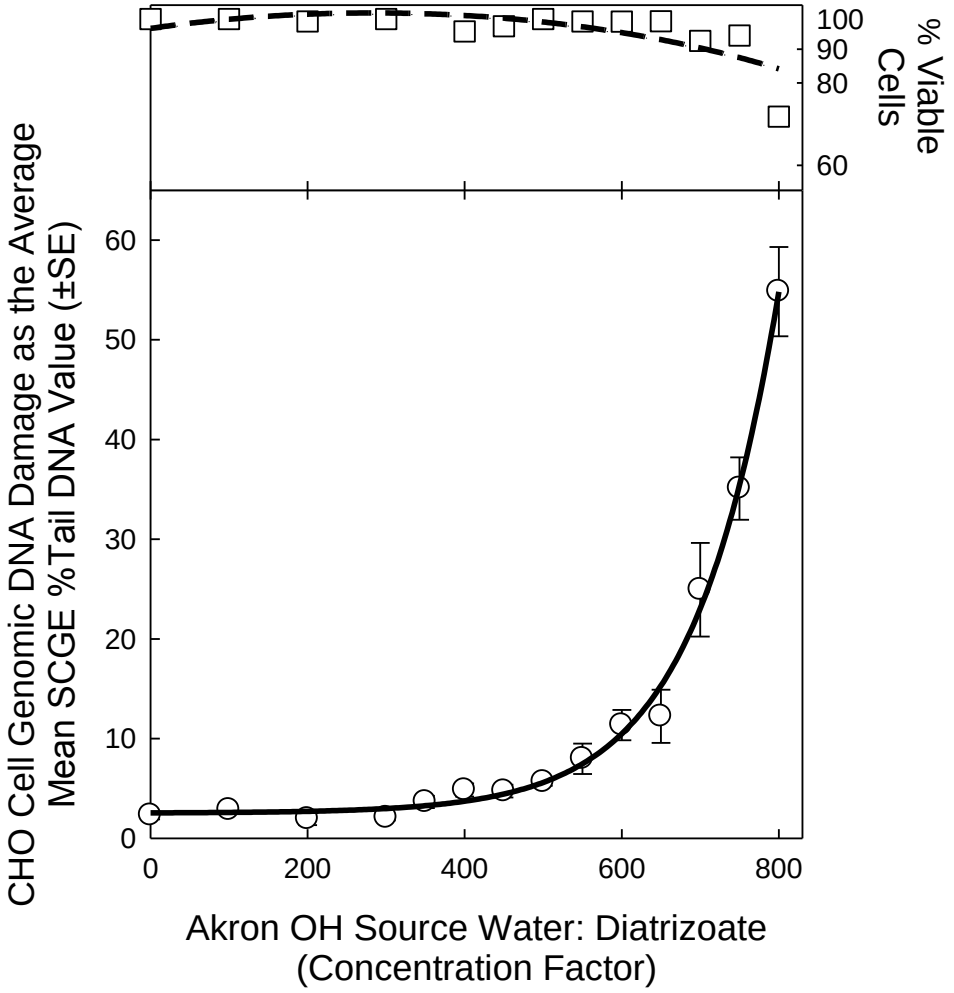


Figure 6.32. CHO cell acute genotoxicity concentration-response curve of AOHTOX-Diatrizoate.

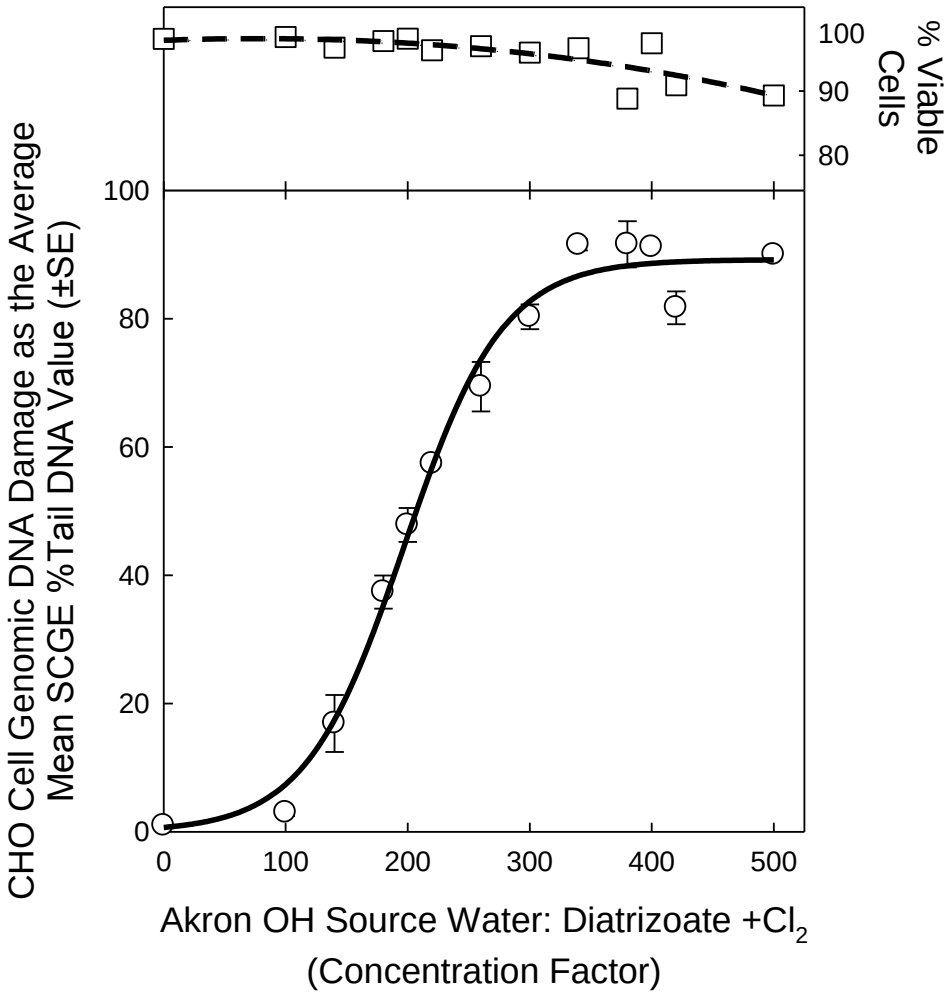


Figure 6.33. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL-Diatrizoate.

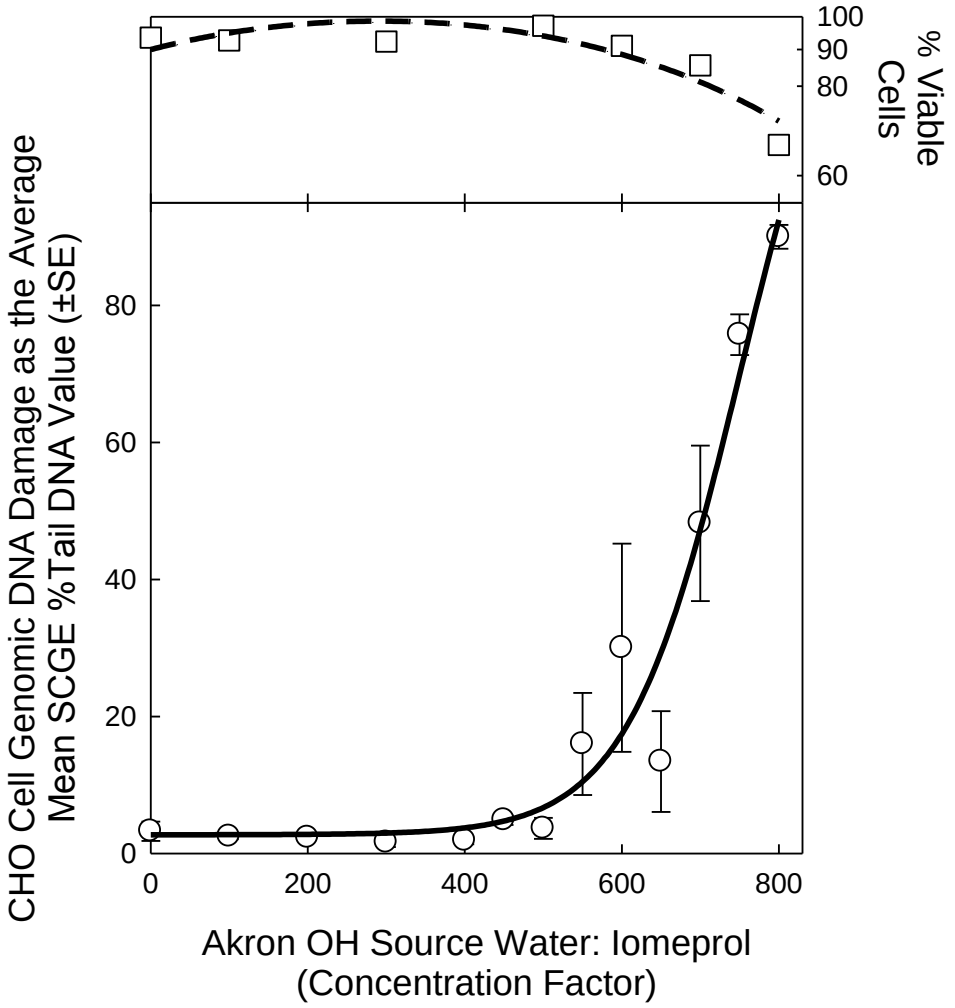


Figure 6.34. CHO cell acute genotoxicity concentration-response curve of AOHTOX-Iomeprol.

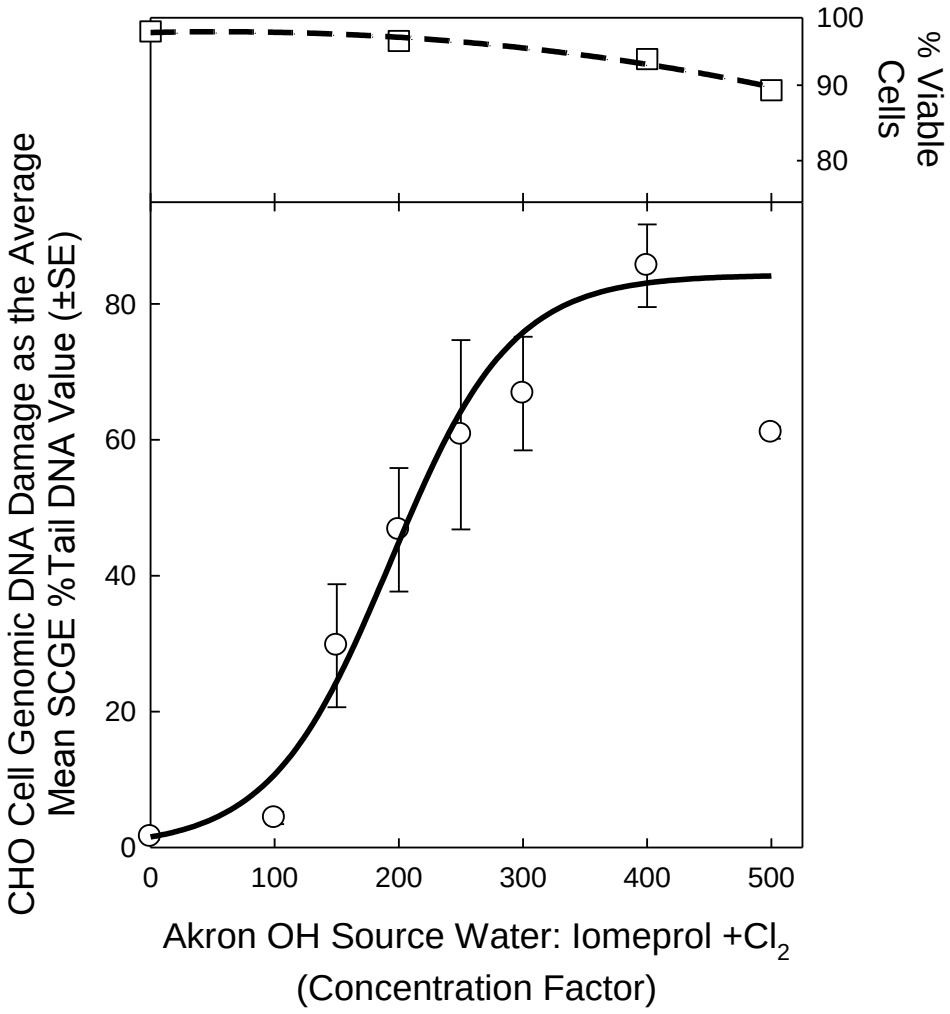


Figure 6.35. CHO cell acute genotoxicity concentration-response curve of AOHTOX-CL-Iomeprol.

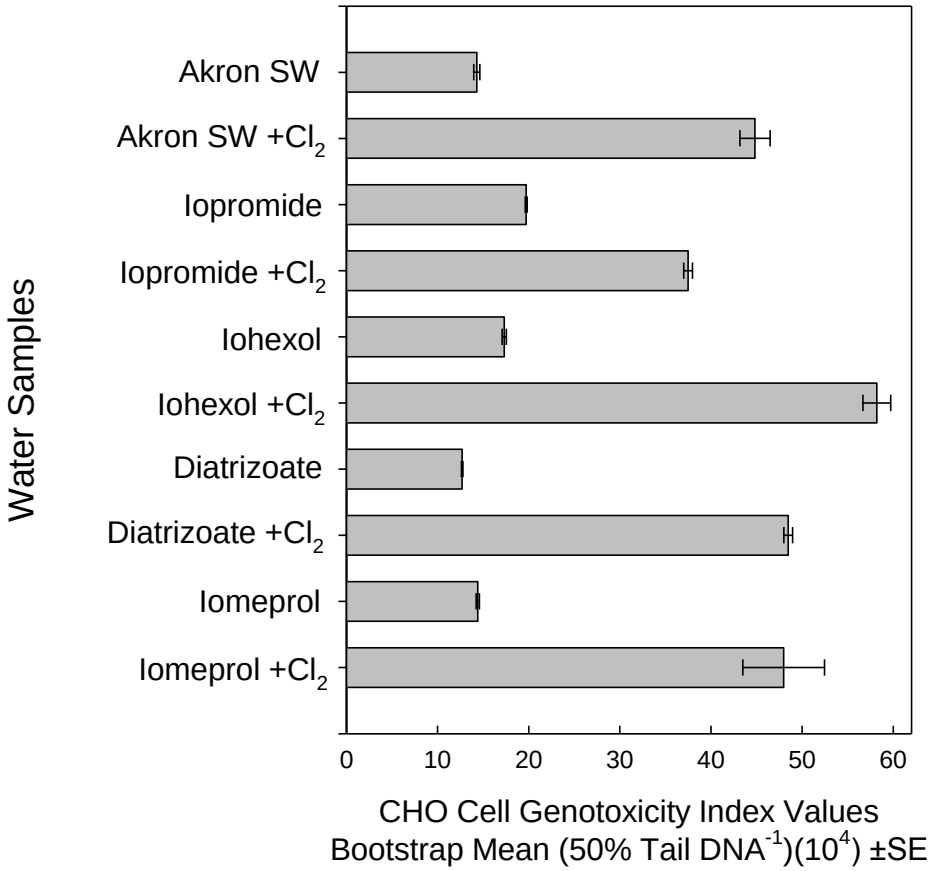


Figure 6.36. Experimental group 2: CHO cell acute genotoxicity index (GTI) values.

CHAPTER 7

CONCLUSIONS

The objectives of this dissertation were to analyze the comparative toxicity of emerging haloacetaldehyde (HAL) drinking water disinfection by-products (DBPs) and to investigate the molecular mechanism of DBP induced toxicity with haloacetic acids (HAAs). Development of a single well microplate-based ATP-protein measurement assay with HAAs, as a novel toxicity metric for DBPs was included as a part of the research. In addition, two complex DBP mixture studies were conducted. The first study investigated the occurrence and *in vitro* mammalian cell toxicity of DBPs in European drinking water samples collected from the site where epidemiological studies on reproductive outcomes were being conducted in relation with the HIWATE project. The correlations of analytical chemistry, analytical biology, and epidemiology were investigated. The second study focused on the impact of iodinated X-ray contrast media in the source water and the type of disinfectant on the overall toxicity of DBP mixtures.

Following conclusions were derived from these studies:

1. Occurrence and comparative toxicity of the haloacetaldehyde disinfection by-products in drinking water

- Iodoacetaldehyde was identified as a new HAL DBP by applying a new analytical chemical method.

- Ten HALs were analyzed for their *in vitro* cytotoxicity and genotoxicity in Chinese hamster ovary (CHO) cells. The most cytotoxic HALs were tribromoacetaldehyde (TCAL) and chloroacetaldehyde (CAL), followed by dibromoacetaldehyde (DBAL) and bromochloroacetaldehyde (BCAL). The most genotoxic HAL was bromoacetaldehyde (BAL), followed by CAL and dibromochloroacetaldehyde (DBCAL).
- HALs were the second most cytotoxic DBP class among six DBP chemical classes (THMs, HAAs, HALs, halonitromethanes, haloacetonitriles, and haloacetamides). Therefore, HALs may adversely affect the public health and the environment.

2. Investigate the biological mechanism induced by haloacetic acid disinfection by-products and the development of a single well microplate-based ATP-protein measurement assay

- All three mono-halogenated HAAs (monoHAAs) including chloroacetic acid (CAA), bromoacetic acid (BAA), and iodoacetic acid (IAA) induced ATP depletion in CHO cells, and simultaneous treatment with 10 mM pyruvate caused a significant recovery of cellular ATP levels.
- The monoHAA-mediated ATP depletion followed a rank order of IAA > BAA >> CAA. This pattern and magnitude of ATP depletion directly correlated with the α-carbon-halide (αC-X) bond length and relative alkylation potential of each monoHAA and was inversely correlated with the αC-X bond dissociation energy.
- ATP depletion was highly correlated with the inhibition kinetics of GAPDH and with diverse measurements of toxicity including cytotoxicity, genotoxicity, mutagenicity and teratogenicity published in the literature over the past decades.

- When the single well microplate-based ATP-protein measurement assay was applied, the cellular ATP levels for monoHAA-treated cells were significantly reduced, but were not concentration-dependent unlike the results from the parallel plate method used for the pyruvate supplementation study.
- With the single well assay, monoHAAs showed the greatest reduction in ATP levels, whereas diHAAs showed a moderate reduction with higher concentration ranges. TriHAAs increases ATP levels. The average ATP levels as the % of negative control were 40.9% for monoHAAs, 72.9% for diHAAs, and 120% for triHAAs.

3. The occurrence and toxicity of disinfection by-products in European drinking waters in relation with the HIWATE epidemiology study

- Eleven drinking water samples were collected from seven cities within five European countries (France, Lithuania, Spain, Italy, and the United Kingdom), where an epidemiologic study of reproductive outcomes was being conducted. Over 90 DBPs were identified in the samples, including several haloacids, halophenols, haloamides, halonitromethanes, haloketones (HKs), haloaldehydes, and haloalkenes.
- Twenty-one target DBPs, including 4 U.S-regulated trihalomethanes (THMs), 9 HAAs, 4 haloacetonitriles (HANs), 2 HKs, trichloroacetaldehyde (chloral hydrate), and trichloronitromethane (chloropicrin) were quantified, and substantial differences were observed in the DBP occurrence from the different locations.

- CHO cell chronic cytotoxicity was analyzed for each HIWATE sample. Based on LC_{50} values, samples from Barcelona, Spain were ranked as the three most cytotoxic (sample 1, 2, and 3).
- CHO cell acute genotoxicity was analyzed for each HIWATE sample. Based on 50% Tail DNA values, the sample from Bradford, U.K. (sample 10) was most genotoxic followed by samples from Kaunas, Lithuania (sample 4) and Valencia, Spain (sample 7).
- The cytotoxic potency index values significantly correlated with the number of identified DBPs ($r = 0.78$; $P \leq 0.005$) and the level of 21 target DBPs ($r = 0.77$; $P \leq 0.006$). The genotoxic potency index values were not significantly correlated with either of these metrics or with any DBP chemical class.
- Cytotoxicity was significantly correlated with the relative concentrations of THMs ($r = 0.74$; $P \leq 0.01$), haloacids ($r = 0.75$; $P \leq 0.008$), other monoacids ($r = 0.68$; $P \leq 0.021$), halodiacids ($r = 0.80$; $P \leq 0.003$), haloamides ($r = 0.68$; $P \leq 0.021$), haloaromatics ($r = 0.64$; $P \leq 0.035$), brominated ($r = 0.68$; $P \leq 0.022$), chlorinated ($r = 0.78$; $P \leq 0.005$) , and iodinated ($r = 0.82$; $P \leq 0.002$) DBPs. There were no statistically significant correlations with genotoxicity and the above DBP classes.

4. The impact of X-ray contrast agents on formation and toxicity of disinfection by-products in drinking water

- Iopamidol generated an enhanced level of CHO cell cytotoxicity and genotoxicity in conjunction with either chlorine or chloramines disinfection. The relative Iopamidol-

mediated increase in CHO cell cytotoxicity and genotoxicity was much greater when chloramine was used as the disinfectant as compared to chlorine.

- Four other ICMs (Iopromide, Iohexol, Diatrizoate and Iomeprol) expressed some cytotoxicity over the control, and expressed higher cytotoxicity when chlorinated. Only Iohexol expressed an enhanced genotoxicity compared to the chlorinated source water control, while Iopromide reduced the genotoxicity.
- Of the five X-ray contrast agents evaluated for their mammalian cell cytotoxicity and genotoxicity, Iopamidol in water disinfected with chlorine or chloramines was clearly the most responsive in generating adverse biological responses.

This study focused on the occurrence and toxicity of emerging HAL DBPs and identified a new HAL DBP, iodoacetaldehyde. This study provided the first systematic, quantitative comparison of HAL toxicity. HALs may adversely affect the public health and the environment and further research are needed to investigate the mode of action of their toxicity. This study extended our research on the molecular mechanism(s) of toxicity of DBPs by investigating the impact of monoHAAs on cellular ATP levels and the attenuation by pyruvate supplementation. This study also provided a platform for the development of a novel bioassay, which quantitatively measures the toxicity of DBPs at molecular cellular levels. An alteration in cellular ATP homeostasis at non-cytotoxic concentrations may be an important metric of toxic action. This assay allows measurements of adverse biological responses at DBP concentrations found in finished drinking water. Additional studies are needed to validate the utility of the assay, and

we will further determine if pyruvate dehydrogenase complex is stimulated by the di- and triHAAs. The HIWATE study was unique in that it integrated quantitative *in vitro* toxicological data with analytical chemistry and human epidemiologic data for drinking water DBPs. Future studies will investigate the possible association between chronic cytotoxicity, acute genotoxicity, multivariate comparisons of identified DBPs and epidemiology across the entire HIWATE program. We plan to compare other *in vitro* and molecular toxicity metrics and rates of adverse pregnancy measurements. The impacts of ICM on complex DBP mixture toxicity were investigated in this study. For precise interpretation, high molecular weight DBPs and low molecular weight DBPs and the ICM transformation products will need to be identified and the toxicity of those individual agents need to be determined.